DEVELOPMENT OF AN INTRAVASCULAR BIOSENSOR FOR PLASMA GLUCOSE MONITORING DURING HEMORRHAGIC SHOCK

FINAL PROGRESS REPORT

by

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The main objective of this research project was the development of an intravenous glucose sensor which can be used to monitor and therapeutically prevent the onset of hypoglycemia in acute hemorrhagic shock. It has been shown that infusion of glucose at a rate sufficient to prevent the fall in glucose level slows or even arrests the decompensatory phase which will enhance the survival rate of wounded soldiers in battlefield settings. The extension of the compensatory phase is also of obvious utilization in civilian trauma management.

The goal of this project was to develop biosensor technology to better define hemorrhagic shock processes in order to optimize fluid resuscitation in trauma victims. Under this project The University of New Mexico studied a series of enzyme electrodes and biosensors, including a miniature needle-sized glucose biosensor suitable for intravenous glucose monitoring. The prototype of the biosensor developed shows a sufficient sensitivity over a wide range of linear response to glucose concentration, which covers the hypo- and hyperglycemic conditions observed during the phases of hemorrhagic shock. The sensor was tested *in vitro* in model buffer solutions, blood plasma and whole blood, demonstrating response characteristics allowing *in vivo* glucose monitoring.

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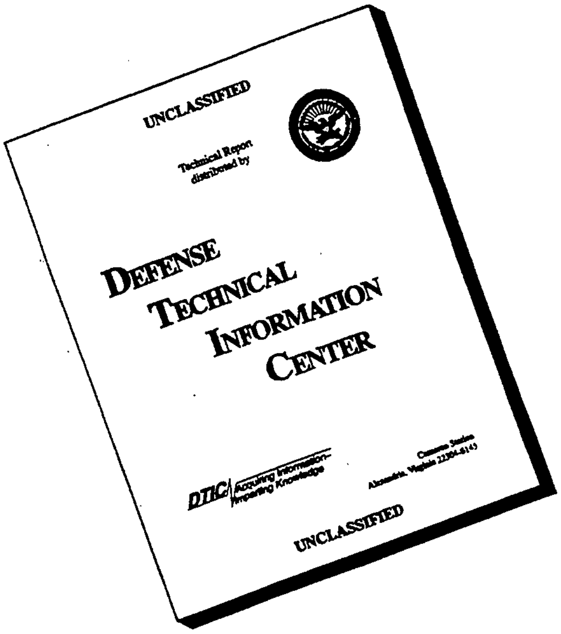
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1. FOREWORD

The main objective of this research project was the development of an intravenous glucose sensor which can be used to monitor and therapeutically prevent the onset of hypoglycemia in acute hemorrhagic shock. The importance of maintaining adequate plasma glucose has been demonstrated clearly in isobaric models of hemorrhagic shock in the research conducted at Walter Reed Army Institute of Research (Washington, DC). The investigations of the Dept. of Experimental Surgery WRAIR demonstrated that (a) fasting abolishes the compensatory hyperglycemia and decreases the survival time in hemorrhagic shock, (b) the onset of vascular decompensation is closely associated with the waning of the hyperglycemia which follows the compensatory phase, and (c) infusion of glucose at a rate sufficient to prevent the fall in glucose level slows or even arrests the decompensatory phase. Extending the compensatory phase is a major goal of the Army Combat Casualty Care Program as it will enhance the survival rate of wounded soldiers in battlefield settings. The extension of the compensatory phase is also of obvious utilization in civilian trauma management.

The goal of this project was to develop biosensor technology to better define hemorrhagic shock processes in order to optimize fluid resuscitation in trauma victims. Under this project The University of New Mexico studied a series of enzyme electrodes and biosensors, including a miniature needle-sized glucose biosensor suitable for intravenous glucose monitoring. The prototype of the biosensor developed shows a sufficient sensitivity over a wide range of linear response to glucose concentration, which covers the hypo- and hyperglycemic conditions observed during the phases of hemorrhagic shock. The sensor was tested *in vitro* in model buffer solutions, blood plasma and whole blood, demonstrating response characteristics allowing *in vivo* glucose monitoring.

Researchers at the University of New Mexico are thankful to the U.S. Army Research Office for the financial support of this project.

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2. LIST OF APPENDIXES

APPENDIX 1.	Abdel-Hamid, I., Atanasov, P., and Wilkins, E. Development of a Needle-type Glucose Biosensor *Analytical Letters*, 27, 1453-1473, 1994
APPENDIX 2.	Abdel-Hamid, I., Atanasov, P., and Wilkins, E. Needle-type Glucose Biosensor with Electrochemically Deposited Enzyme in a Platinum Black Matrix *Electroanalysis*, 7/8, 738-741, 1995
APPENDIX 3.	Abdel-Hamid, I., Atanasov, P., and Wilkins, E. Development of a Needle-type Biosensors for Intravascular Glucose Monitoring *Analytica Chimica Acta*, 313, 45-54, 1995
APPENDIX 4.	Yang, Q., Atanasov, P., and Wilkins, E. Needle-type Sensors for Whole Blood Glucose Monitoring *Biomedical Instrumentation and Technology*, submitted 1996 (manuscript)
APPENDIX 5.	Atanasov, P., Gamburzev, S. and Wilkins, E. Needle-type Glucose Biosensors Based on Pyrolyzed Cobalt-tetramethoxy-phenilporphyrin Catalytic Electrode *Electroanalysis*, in press 1996 (galley proof)
APPENDIX 6.	Yang Q., Atanasov, P., Wilkins, E. and Hughes R.C. Enzyme Electrodes with Glucose Oxidase Immobilized on Stöber Glass Beads *Analytical Letters*, 28, 2439-2457, 1995
APPENDIX 7.	Krishnan R., Atanasov, P., and Wilkins, E. Mathematical Modeling of an Amperometric Enzyme Electrode Based on a Porous Matrix of Stöber Glass Beads *Biosensors and Bioelectronics*, in press 1996 (manuscript)

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4. BODY OF REPORT

A. STATEMENT OF THE PROBLEM STUDIED

The development of an indwelling glucose biosensor for short-term continuous *in vivo* monitoring of glucose concentration in blood should lead to a marked improvement in the clinical management and scientific understanding of hemorrhagic shock. The availability of such a sensor could provide the means to monitor blood glucose levels in real time, thereby aiding the maintenance and monitoring of patients in hemorrhagic shock, and hence increasing the survival rate. In addition, a continuous *in vivo* sensor would make it far more feasible to conduct studies to determine what influences fluctuations of blood glucose concentration may have on the survival of patients in hemorrhagic shock.

The goal of this project was to develop biosensor technology to better define hemorrhagic shock processes in order to optimize fluid resuscitation in trauma victims. Specific aims of the research include the determination of the short-term life of the biosensor *in vivo*; improvement of the reliability and reproducibility of the sensor response, the sensor response time; the characterization of the biosensor behavior and its biocompatibility in the body fluid environment (including possible interference effects from various body chemicals).

Relevance to the U.S. Army Missions

WRAIR COMBAT CASUALTY CARE PROGRAM - Need for Glucose Sensor

General Problem:	Delayed delivery to definitive care leads to diminished ability to resuscitate due to decompensation.
Mission:	Develop resuscitation strategies that will delay the onset of the vascular decompensation and prolong the tolerable hypovolemic period.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

Glycaemia and the Hemorrhagic Shock

A recent biomedical application of glucose sensors is in the management of hemorrhagic shock. Continuous on-line monitoring of glucose concentration appears be of crucial importance in the management of trauma cases involving extensive bleeding - hemorrhage. Hemorrhagic shock generally consists of two phases, the compensatory phase and the decompensatory phase. Hyperglycemia is associated with a deterioration of mechanisms which support homeostasis in the decompensatory phase of hemorrhagic shock. Plasma glucose has been shown to follow a certain pattern during hemorrhagic shock. At the onset of the hemorrhage, the plasma glucose level rises to hyperglycemic levels in 2 hours (decompensatory phase). This is followed by the compensatory phase in which plasma glucose levels start to fall, reaching hypoglycemic levels. It has also been demonstrated that early glucose administration to maintain the hyperglycemic phase arrests decompensation and prolongs survival. The compensatory phase of hemorrhagic shock lasts for approximately 2 hours, making the time delay and accuracy of subcutaneous monitoring of glucose levels unacceptable. Thus, hemorrhagic shock requires direct monitoring of glucose in the bloodstream by means of an intravascular sensor for maximum accuracy and fast response. The importance of glucose monitoring during hemorrhagic shock lies in increasing the survival rate of injured patients while they are being transported to a hospital. Unlike diabetes, effective treatment of hemorrhagic shock requires direct intravascular implantation of sensors and continuous monitoring of glucose levels for short periods of time (up to several hours).

A needle-type glucose biosensor has been developed for intravascular monitoring of glucose levels in accident victims under risk of hemorrhagic shock. The main method for the construction of this glucose sensor is based on the use of enzyme electrodes employing Glucose Oxidase enzyme. Amperometric measurement has been chosen as it provides a linear dependence of the signal versus analyte concentration. The research and development undertaken at the University of New Mexico towards needle-type glucose biosensor for intravascular applications can be divided into three branches:

- Engineering and development of sensor prototypes based on platinum amperometric transducer for hydrogen peroxide oxidation incorporated into a hypodermic stainless steel needle. Three different sensor prototypes have been developed differing in the immobilization technique for Glucose Oxidase. The first sensor prototype employs Glucose Oxidase enzyme which is immobilized on modified carbon powder in a gel matrix. Polycarbonate membranes that are either untreated or treated with Nafion or Silastic have been used as external diffusion limiting membranes. The second sensor prototype was constructed employing the electrophoretic deposition of the Glucose Oxidase enzyme in an electrochemically grown platinum black matrix. The last sensor prototype was constructed with the Glucose Oxidase enzyme entrapped in a poly(1,3-phenylenediamine) film. Polyurethane, cellulose acetate or polyvinylchloride layers were used as an outer glucose diffusion and protective membrane. The last prototype was used as a basis for the development of a whole blood compatible sensor for intravascular glucose monitoring (See APPENDIXES 1-4).

- Investigation and initial steps towards development of amperometric needle-type biosensors employing non-platinum electrocatalysts based on pyrolyzed N_4-metal chelates. These biosensors are based on preparation of the working electrode with catalyst and adsorbed enzyme (an enzyme electrode) from a pressed matrix (tablet) of teflonized (PTFE treated) carbon black. This technique allows manufacturing numerous sensors from the same pressed tablet, all of which demonstrate good reproducibility of their parameters (sensitivity, linear range, lifetime). The approach has been demonstrated by using Glucose Oxidase enzyme in construction of glucose needle-type sensor (See APPENDIX 5).

- Investigation of novel sensor materials and technologies with potential in future sensor miniaturization and microfabrication. Sol-gel techniques for controlled fabrication of porous coatings are currently attracting an increasing attention. Experimental evaluation and theoretical description of a miniature Stöber glass-coated enzyme electrode for glucose needle-type biosensor applications was undertaken (See APPENDIXES 6 and 7).

Development of a Needle Glucose Sensor Prototype

Three different sensor prototypes have been developed differing in the immobilization technique for Glucose Oxidase. Various tests were conducted to evaluate the sensors performance *in vitro*. These tests include the measurement of glucose levels in buffer solutions, evaluation of the effect of five potential physiological interferents on sensor performance, undiluted blood plasma tests, whole blood *in vitro* tests, a hemorrhagic shock simulation and life time tests.

The First sensor prototype employs Glucose Oxidase enzyme which is immobilized on modified carbon powder in a gel matrix. The sensor employs polycarbonate membranes that are either untreated or treated with Nafion or Silastic, and Glucose Oxidase enzyme which is immobilized on modified carbon powder in a gel matrix. Sensor performance was evaluated *in vitro* and the sensor showed a sensitivity of up to 10 nA/mM and a linear range of up to 30 mM.

Evaluation of the sensor response in serum showed similar sensitivity and linear range as obtained using calibration curves in buffer solution. The sensors were operated continuously for 28 days in phosphate buffer solution and no significant change in the sensitivity and the linear range was observed during the first 7 days. Sensors show a minimum change in their performance when stored inactive in buffer at 4°C for at least eight weeks (APPENDIX 1).

The Second sensor prototype employs an immobilization technique based on the electrophoretic deposition of the Glucose Oxidase enzyme in an electrochemically grown platinum black matrix. The platinization and electrophoretic incorporation of the enzyme particles was carried in a solution containing 33 mg of sodium hexachloroplatinate and 30 mg of Glucose Oxidase and 0.6 mg of lead acetate in one mL at pH 3.5. This preparation process was performed potentiostatically at -0.2 V versus a silver/silver chloride reference electrode. The sensor was coated with Nafion to decrease effect of interferents. These tests include the measurement of glucose levels in buffer solutions containing various potential physiological interferents, as well as in bovine serum. Sensors have a large linear range (up to 33 mM) and fast response. A Nafion coating diminished the sensor response to common interferents by at least 50%. Sensors were operated for several weeks being stored at 4 °C between measurements. Sensors operated well in undiluted blood plasma showing close correlation with response in buffer solution. The advantages of this technique is its simplicity and high controllability (APPENDIX 2).

The Third sensor prototype employs polyurethane, cellulose acetate or PVC layers as an outer glucose diffusion and protective membrane, and Glucose Oxidase enzyme entrapped in poly(1,3-phenylenediamine) film. The electropolymerization of 1,3-phenylenediamine and the incorporation of the enzyme particles was carried in a solution containing 3-5 mg of 1,3-phenylenediamine, 20 mg of Glucose Oxidase and 20 mg of enzyme immobilized on ULTI carbon powder in 9 mL of phosphate buffer solution (pH 7.4) together with 1 mL of Nafion solution. This preparation process was performed potentiostatically at +0.65 V versus a silver/silver chloride reference electrode for 15 minutes. Another layer of poly(1,3-phenylenediamine) was grown over the sensor from a solution containing 5 mg of 1,3-phenylenediamine for 10 minutes. A variety of different polymer coatings were employed in order to attempt to extend the linear range of the prepared sensors. Different concentrations of polyurethane, polyvinylchloride (PVC), and cellulose acetate coating solutions were investigated. The polymer coatings were obtained by dipping the face of the sensor in the polymer solution. Sensor performance was evaluated *in vitro* and the sensor shows a sensitivity of up to 35 nA/mM and a linear range of up to 38 mM. Evaluation of the sensor response in serum showed similar sensitivity and linear range as obtained using calibration curves in buffer solution. The sensor has a short response time of 23 seconds.

The sensors were operated continuously for 14 days in phosphate buffer solution, and no significant change in the sensitivity and the linear range was observed during the first 5 days. Sensors show a minimum change in their performance when stored inactive in buffer at 4°C for at least eight weeks (APPENDIX 3).

The performance of each of the three sensor prototypes was suitable for the proposed application in varying degrees. **Table 1** presents a comparison of the performance of each of the three sensor prototypes based on the immobilization of Glucose Oxidase in a gel matrix, a platinum black matrix or a poly(1,3-phenylenediamine) film. The ultimate goal of the sensor development will be its use in intravascular glucose monitoring and especially in victims in risk of hemorrhagic shock. Thus, a comparison of the performance of each of the three sensor prototypes and how well they satisfy the constraints set forth by this goal would be helpful in evaluating which sensor prototype will be used.

Hemorrhagic shock is characterized by the alteration of glucose concentrations between the hypoglycemic and hyperglycemic levels. Hence a linearity of the sensor signal versus the glucose concentration is required. This linearity should be at least 22 mM (400 mg/dL) to assure linear output of the sensor within some safety margin. It can be seen from **Table 1** that the First sensor prototype (with enzyme immobilized on carbon powder suspended in a gel matrix) satisfies this requirement by the use of Nafion or Silastic coated polycarbonate membranes. The linearity of the sensor output signal versus the glucose concentration is extended to 22 mM with a sensitivity of 8.46 nA/mM and 27.7 mM with a sensitivity of 6.48 nA, by the use of Nafion and silastic coated polycarbonate membranes respectively. Yet, the untreated polycarbonate membrane coated sensor showed a linearity of up to 13.3 mM, clearly less than that required, and hence will not be considered further in the discussion. The Second and Third sensor prototypes satisfied this requirement. The Second sensor prototype (with enzyme entrapped in a platinum black matrix) showed a linearity of up to 33 mM. While the Third sensor prototype showed a linearity of 26.7 mM with sensitivity of 1.62 nA/mM, 31.1 mM with a sensitivity of 1.35 nA/mM, and 37.7 mM with a sensitivity of 1.8 nA/mM for cellulose acetate (CA), polyurethane (PU) and polyvinylchloride (PVC) coated sensors, respectively.

Another requirement for monitoring of glucose levels during hemorrhagic shock is a fast sensor response (short response time) to changes in glucose concentration. Comparing the response times of the three sensor prototypes, the First sensor prototype shows response times of 300 and 480 seconds for Nafion and silastic coated polycarbonate covered sensors. The Second sensor prototype shows response time of 60 seconds. while the Third sensor prototype shows response times of 135, 24 and 35 seconds for CA, PU and PVC coated sensors.

Another test reflecting the response times of the sensors is the *in vitro* hemorrhagic shock simulation test. This test reflects the response times of the sensors while responding to large changes in glucose concentration (between hypoglycemic and hyper glycemic levels). **Table 1** shows the First sensor prototype in the case of Nafion coated PC membrane to have a response time of 16 minutes for the increasing and 22 minutes for the decreasing step. While the silastic coated PC membrane covered sensor shows a response time of 25 minutes for the increasing and 35 minutes for the decreasing step.The Second sensor prototype shows a response time of 195 seconds for the increasing and 216 seconds for the decreasing step changes. The Third sensor prototype having an outer PVC membrane shows a response time of 104 seconds for the increasing and 306 seconds for the decreasing step. It is thus seen that the Second and Third sensor prototypes show the lowest response times in response to both small and large changes in glucose concentration.

The reproducibility of the sensor signal is good in all three sensor prototypes. The variations in the sensor response does not exceed 5% in the First and Third sensor prototypes response, while is less than 10% for the Second sensor prototype.

The effect of physiological interferents on the sensor signal is also another important aspect to be considered as the sensor will be implanted in the body. The effect of interferents was tested by five common substances. The First sensor prototype shows a decrease in sensitivity after the addition of interferents. Nafion coated polycarbonate membrane for this prototype showed a decrease in the sensor response to glycine, urea and acetaminophen. The Second sensor prototype shows a decreased effect of glycine, urea and ascorbic acid. There is marked effect of uric acid on the sensor signal as well as a decrease in sensor sensitivity. While the Third sensor prototype completely eliminates the effects of glycine and urea while greatly reducing the effect of uric and ascorbic acid. There is also no decrease in sensor sensitivity as a result of adding the interferents.

In vitro tests in undiluted blood plasma try to simulate the working environment in the body. All three sensor prototypes responded well in plasma showing close correlation with their performance in buffer. Some decrease of sensitivity may be observed in the case of the Second and Third sensor prototypes. This is expected due to adsorption of proteins on the sensor surface. No decrease in sensitivity is observed for the First sensor prototype.

All three prototypes showed a life time of at least one week with no significant change in the sensor performance hence satisfying the life time requirements.

One last aspect to be considered is the ease of fabrication of the sensor prototypes. In general, the three sensor prototypes are easily fabricated, with the First sensor prototype being fabricated in no more than three hours. The Second and Third sensor prototypes are fabricated in one and half hours. The Third sensor prototype has the advantage of electrochemical growing of the entrapping film hence allowing more controllability on the thickness of the film and hence the amount of enzyme entrapped.

Table 1. A comparison of the performance of the three developed sensor prototypes with the different external polymer membranes.

Performance	First Prototype	Second Prototype	Third Prototype
Linearity (mM)	PC: 13.3 N PC: 22.2 S PC: 27.7	33	CA: 26.7 PU: 31.1 PVC: 37.7
Sensitivity (nA/mM)	PC: 9.9 N PC: 8.46 S PC: 6.48	36	CA: 1.62 PU: 1.35 PVC: 1.80
Response Time (seconds)	PC: 180 N PC: 300 S PC: 480	60	CA: 183 PU: 24 PVC: 35
Hemorrhagic Shock: Duration of Increasing Glucose Step (minutes)	PC: 16 N PC: 16 S PC: 25	5	PVC: 1.7
Duration of Decreasing Glucose Step (minutes)	PC: 20 N PC: 22 S PC: 35	10	PVC: 5.1
Signal Variation	< 5%	< 10%	< 5%
Effect of Interferents	Decreased Sensitivity	Decreased Sensitivity	No change in sensitivity
Plasma Test	No change in sensitivity	Decreased Sensitivity	Decreased Sensitivity

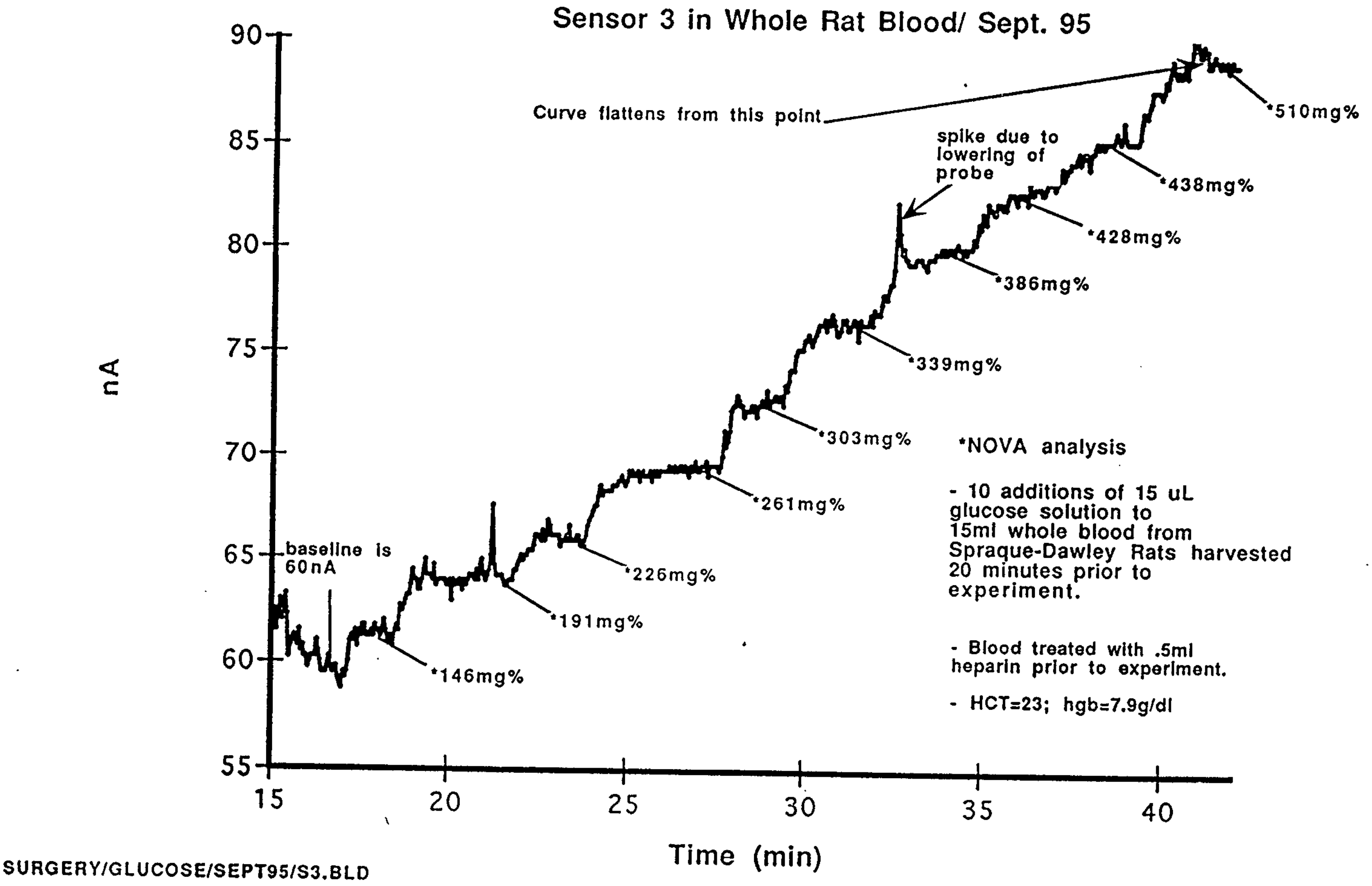
PC: polycarbonate
CA: cellulose acetate
PU: polyurethane
N PC: Nafion coated polycarbonate
S PC: silastic coated polycarbonate

A comparison of the performance of each of the three sensor prototypes showed the First sensor prototype to have a longer response time relative to the Second and Third prototypes. Also the Second sensor prototype has more response to interfering substances relative to the Third sensor prototype. Hence, the Third sensor prototype employing the phenylenediamine film was more suited for application in hemorrhagic shock.

Continuation of this work includes the evaluation of needle-type glucose biosensor (based on the Thirds sensor prototype) performance in whole blood as a final test before its *in vivo* testing. A new surface process technology employing electrochemical fixation of a bioactive substance (enzyme and heparin) to a sensor electrode was developed to provide biocompatibility and functionality. The fabrication process includes electro-entrapment of glucose oxidase and heparin on a platinum electrode by using 1,3-phenylenediamine co-deposition. The process of electrochemical heparinization was carried out in 20 ml buffer solution (pH 7.4) containing 30 mg of 1,3-phenylenediamine and 2.5 mL heparin potentiostatically at +0.7 V versus a silver/silver chloride reference electrode for 10 minutes. This procedure forms the heparin layer over the carbon-enzyme layer on the electrode surface. PVC was used as the outer coating in order to extend the linear range. The sensors show a sensitivity of 3 nA/mM and a linear range up to 25 mM at 37 °C. when tested in whole blood. The sensors are characterized by a fast response. When tested at physiological glucose levels, the sensors demonstrate satisfactory low interference from common interfering substances. This technology seems very promising for the preparation of future implantable intravascular biosensor. The electrochemical-entrapment immobilization of heparin, useful as a thromboresistant coating, and the immobilization of the glucose oxidase onto a platinum electrode surface, was demonstrated. This technology can greatly improve the sensor response to glucose in the whole blood and may be applied to other devices for intravascular applications (See APPENDIX 4).

During the project period small series (from 4 to 6 samples in each) of the various constructions of needle-type glucose biosensors has been sent to Walter Reed Army Institute of Research, Division of Experimental Surgery, for independent evaluation and characterization. These sensors were subject of the same in vitro tests as performed at the University of New Mexico. In most of the cases the results obtained at Walter Reed Army Institute of Research coincide with those obtained in our laboratory. **Figure 1** shows a recording obtained during a whole blood test at 37 °C of a heparinized needle-type glucose sensor conducted at Walter Reed Army Institute of Research. The initial blood glucose level is measured to be 85 mg/dL (by a reference method). After every addition, the concentration of bulk glucose was increased by 40 mg/dL.

From **Figure 1** it can be seen that sensor responded to the glucose concentration increase in whole blood. After testing in whole blood, the electrodes were washed and then tested again in buffer solution. It was found that the sensitivity of the sensors remained the same as before the test in the whole blood. This demonstrated that the electrode surface has not been irreversibly clogged by the blood albumin. The sensor signal in whole blood however, has of lower amplitude than that obtained in buffer solution. This could be an indication that there is some adsorption of substances (proteins, lipids) on the electrode surface acting as an additional diffusion barrier for glucose and diminishing the sensor signal.



Sensor 3 in Whole Rat Blood/ Sept. 95

*NOVA analysis

- 10 additions of 15 uL glucose solution to 15ml whole blood from Sprague-Dawley Rats harvested 20 minutes prior to experiment.
- Blood treated with .5ml heparin prior to experiment.
- HCT=23; hgb=7.9g/dl

SURGERY/GLUCOSE/SEPT95/S3.BLD

Figure 1. Recording of the heparinized needle-type glucose sensor signal obtained during a glucose concentration test in whole blood at 37 °C. The test was conducted at Walter Reed Army Institute of Research.

Needle-type Glucose Biosensors with Non-platinum Electrocatalyst

Amperometric needle-type glucose biosensors employing non-platinum catalytic electrodes made from pyrolytic electrode of pyrolyzed cobalt-tetramethoxy-phenylporphyrin (CoTMPP) has been investigated. A technique for preparation of the working electrode with catalyst and adsorbed enzyme (enzyme electrode) from a pressed matrix (tablet) was developed. This technique allows manufacturing numerous sensors from the same pressed tablet, all of which demonstrate good reproducibility of their parameters (sensitivity, linear range, lifetime).

The catalytic electrode was fabricated in the form of a pressed tablet, from which a disc-shaped segment was placed inside a flattened needle tip. This procedure consists of four consecutive steps: (a) formation of a carrier tablet from teflonized (PTFE treated) carbon black, (b) formation of the catalytic layer over the pressed carbon black matrix, (c) enzyme deposition (immobilization) on the catalytic layer and (d) cutting a cylindrical segment from the tablet to obtain the enzyme electrode. The carrier layer was made of teflonized carbon black pressed at 260 KPa to give a electro-conductive matrix. The matrix had a disk shape with 10 cm^2 area and thickness about 0.4 mm. After the formation of the carbon black matrix, the dispersed catalyst from pyrolyzed CoTMPP was poured on one of the matrix sides (c.a. 0.5 mg/cm^2) and pressed at 65 KPa to form a thin layer. The total thickness of the tablet after adding the catalytic layer did not exceed 0.5 mm. The resulting tablet is sufficiently elastic and durable to be stored or transported.

In general, electrodes of different shapes and sizes can be cut out from such a tablet. Macroelectrodes (disk-shaped, rectangular pieces or strips of several cm^2) as well as microelectrodes (usually disks with diameters between 0.5 mm and 1 mm) can be fabricated. The reproducibility of this technique is high and relies on the reproducible properties of the teflonized carbon black developed initially for the needs of the battery technology.

The side of the tablet with the supported pyrolyzed CoTMPP (catalytic layer) was impregnated with enzyme solution containing 20 mg/cm^3 Glucose Oxidase in 0.5% (w/v) Nafion in phosphate buffer solution. The enzyme/Nafion layer was then dried at room temperature for two hours forming an active layer of immobilized Glucose Oxidase. The prepared enzyme layer contained approximately 1 mg/cm^2 Glucose Oxidase and c.a. 0.25 mg/cm^2 Nafion (loaded amount) in the immobilization matrix.

Different glucose diffusion (external) membranes have been studied in order to extend the linear range of the biosensor response. These coatings were made by dipping of the sensor tip in a polymer solution (5 % w/v. of CA in acetone, PU and PVC in tetrahydrofurane). A cellulose paper membrane was used the paper segment is cut out from a plain sheet of white Xerox printer/copy paper during the enzyme electrode preparation. The paper piece is then impregnated with 2 % wt./v. of CA solution in acetone). Linearity of the sensor response to glucose concentrations up to 35 mM was observed when a cellulose paper membrane, impregnated with cellulose acetate was employed. Sensors demonstrated a shelf lifetime of 30 days (with constant parameters of the signal output) and can be used for continuous glucose monitoring *in vitro* for at least 5 days.

The results presented show that a non-platinum catalytic hydrogen peroxide oxidizing electrode can be used successfully in a needle-type glucose biosensor. The proposed technique for enzyme electrode preparation from a pressed multi-layer carbon black tablet provides the possibility to improve the reproducibility of the sensor parameters within a set of sensors (See APPENDIX 5).

Enzyme Electrodes with Glucose Oxidase Immobilized on Stöber Glass Beads

Some efforts were directed towards the development and experimental evaluation of a miniature Stöber glass-coated enzyme electrode for glucose biosensor applications. The enzyme (Glucose Oxidase) was chemically immobilized onto the surface of the Stöber glass beads after a silanization procedure. The sensors were fabricated using glass beads of different radii ranging from 20 nm to 100 nm. The overall thickness of the glass coating was about 1.4 μm in all cases.

Platinum wires were coated with a layer of Stöber glass beads by sol-gel technology using the facilities of Sandia National Laboratories. The porous Stöber glass films were made by multiple dip coating of Pt wires. After each dip the wires were cured at 400°C in air. The monodisperse glass spheres were made by the Stöber method and maintained in ethanol-water (pH 11) solutions. Films made in this manner have a very high free volume and porosity. The perceived advantages of using Stöber glass films for immobilizing bioactive molecules such as enzymes include the rigid structure of the films, strong adhesion to the sensor surface, and high potential diffusion constants for both reactants and products. Other films, both organic polymers and inorganic sol-gel matrices, enmesh the biomolecules in a gel where diffusion constants can be lowered by factors of up to 10^3. It is known from BET type porosity and surface area measurements that Stöber films made from different size spheres have a well defined and reproducible porosity of 37%, which is larger than one would expect from close packing of the spheres.

Silanization was utilized to prepare the Stöber glass beads and the platinum wire surfaces to bond with glutaraldehyde. The glass-coated needle-type electrode was made by using silanization followed by cross-linking the enzyme onto glass beads. Initial calibration curves of the electrodes show an increase in the response value with decrease in the bead radius. Life-time evaluation tests, however, demonstrate a different behavior of the electrodes. At the end of a 50 day test, the electrode with bead radius of 70 nm remained most stable. The response of this electrode was stable over this period of time and no apparent decrease in its sensitivity was observed. This study suggests that there may be a relation between the immobilized enzyme stability and the porosity of the glass-bead layer. Preliminary tests in undiluted blood plasma suggest that electrodes can be used for glucose measurements in body fluids. Further experiments need to be performed to improve the performance of the biosensor. This includes improvement in the control of porosity, surface pretreatment and particularly avoidance of cracking of the Stöber glass layer (See APPENDIX 6).

A mathematical model of a glucose sensor based on the amperometric detection of hydrogen peroxide using immobilized Glucose Oxidase has been developed. In this sensor the enzyme (Glucose Oxidase) was immobilized on Stöber glass beads that were attached to a Platinum electrode. The influence of bead radius (ranging 20 nm, 45 nm, 70 nm, 100 nm and 200 nm) on the performance of the sensor was analyzed. The total enzyme concentration defined per unit interfacial area increased directly with the bead radius and the effective diffusivity of the substrate in the enzyme layer decreased with increasing bead radius. The model described approximate analytical solutions for the behavior of the system, which was assumed to follow Michaelis-Menten scheme of reaction. Two distributions of the enzyme in the bead layer had been taken into consideration in the discussion. Numerical solutions had also been presented to give a complete picture of the behavior of the system. Comparison of the numerical solutions and approximate analytical solutions suggested that the model was consistent in the regions of approximations. The model predicted different behavior of the system on either side of the critical radius (approximately 26 nm). The process is essentially diffusion controlled for the sensors with beads of radius smaller than the critical radius and the current response of the sensors in this case increased with bead radius. The current response of the sensors with beads of radius greater than the critical radius, decreased with the increase in bead radius. The regime of operation (kinetic control or diffusion control) for this case depended on the Thiele modulus (See APPENDIX 7).

LIST OF JOURNAL PUBLICATIONS

ACKNOWLEDGING THE ARMY RESEARCH OFFICE GRANT PR-P-32452-LS-ISP

for the period September 1993 - March 1996

1. Abdel-Hamid, I., Atanasov, P., and Wilkins, E.
   Development of a Needle-type Glucose Biosensor
   Analytical Letters, 27, 1453-1473, 1994

2. Abdel-Hamid, I., Atanasov, P., and Wilkins, E.
   Needle-type Glucose Biosensor with Electrochemically Deposited Enzyme in a Platinum Black Matrix
   Electroanalysis, 7/8, 738-741, 1995

3. Abdel-Hamid, I., Atanasov, P., and Wilkins, E.
   Development of a Needle-type Biosensors for Intravascular Glucose Monitoring
   Analytica Chimica Acta, 313, 45-54, 1995

4. Yang, Q., Atanasov, P., and Wilkins, E.
   Needle-type Sensors for Whole Blood Glucose Monitoring
   Biomedical Instrumentation and Technology, submitted 1996

5. Atanasov, P., Gamburzev, S. and Wilkins, E.
   Needle-type Glucose Biosensors Based on Pyrolyzed Cobalt-tetramethoxy-phenilporphyrin Catalytic Electrode
   Electroanalysis, in press 1996

6. Yang Q., Atanasov, P., Wilkins, E. and Hughes R.C.
   Enzyme Electrodes with Glucose Oxidase Immobilized on Stöber Glass Beads
   Analytical Letters, 28, 2439-2457, 1995

7. Krishnan R., Atanasov, P., and Wilkins, E.
   Mathematical Modeling of an Amperometric Enzyme Electrode Based on a Porous Matrix of Stöber Glass Beads
   Biosensors and Bioelectronics, in press 1996

LIST OF CONFERENCE PRESENTATIONS
PUBLISHED AS ABSTRACTS

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INTERNATIONAL CONFERENCES

1. Abdel-Hamid, I., Atanasov, P., and Wilkins, E.
   Needle-type Glucose Biosensor with Electrochemically Deposited Enzyme in Platinum Black
   ANABIOTEC'94, Fifth International Symposium on Analytical Methods, Systems and Strategies in Biotechnology, Minneapolis, Minnesota, October 31 - November 2, 1994

2. Wilkins, E. , Abdel-Hamid, I. and Atanasov, P.
   Needle-Type Biosensor Employing Polyphenylenediamine as an Entrapping Film
   BCEIA'95 The Sixth Beijing Conference and Exhibition on Instrumental Analysis, Beijing, China. October 24-28, 1995.

3. Atanasov, P., Gamburzev, S. and Wilkins, E.
   New Concept for the Needle-type Biosensors
   BIOSENSORS '96, Fourth World Congress on Biosensors and Bioelectronics, Bangkok, Thailand, May 29-31, 1996

4. Wilkins, E., Abdel-Hamid, I., Yang, Q., Ghindilis, A. and Atanasov, P.
   Needle-Type Sensor for Intravascular Glucose Monitoring During Hemorrhagic Shock
   The 6th International Meeting on Chemical Sensors, National Institute of Standards and Technology, Gaithersburg, Maryland, July 22-25, 1996

NATIONAL MEETINGS

5. Morshed, M., Abdel-Hamid, I., Atanasov, P., and Wilkins, E.
   Development of a Needle-Type Intravascular Glucose Biosensor
   ACS National Meeting, Anaheim, California, April 2-6, 1995

6. Wilkins, E., Yang, Q. and Atanasov, P.
   Needle-Type Intravascular Glucose Biosensor
   AIChE 1995 Annual Meeting, Miami, Florida, November 11-15, 1995
   Biomedical Engineering Session

7. Yang, Q., Atanasov, P., and Wilkins, E.
   Glucose Biosensor Based on Porous Matrix of Stöber Glass Beads
   AIChE 1995 Annual Meeting, Miami, Florida, November 11-15, 1995
   Biomedical Engineering Session

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Graduate Special Problem Report (December 1995):
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DEVELOPMENT OF AN INTRAVASCULAR BIOSENSOR FOR PLASMA GLUCOSE MONITORING DURING HEMORRHAGIC SHOCK

by

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5. APPENDIXES

ANALYTICAL LETTERS, 27(8), 1453-1473 (1994)

DEVELOPMENT OF A NEEDLE TYPE GLUCOSE BIOSENSOR

KEY WORDS: Glucose biosensor, needle sensor, glucose concentration monitoring

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ABSTRACT

A needle type glucose biosensor has been developed for monitoring of glucose levels during hemorrhagic shock. The sensor employs polycarbonate membranes that are either untreated or treated with Nafion or Silastic, and Glucose Oxidase enzyme which is immobilized on modified carbon powder in a gel matrix. Sensor performance was evaluated *in vitro* and the sensor showed a sensitivity of up to 10 nA/mM and a linear range of up to 30 mM. Evaluation of the sensor response in serum showed similar sensitivity and linear range as obtained using calibration curves in buffer solution. The sensors were operated continuously for 28 days in phosphate buffer solution and no significant change in the sensitivity and the linear range was observed during the first 7 days. Sensors show a minimum change in their performance when stored inactive in buffer at 4°C for at least eight weeks.

INTRODUCTION

A substantial amount of research has been devoted to the development of sensors for analytical purposes in medicine and biology [1]. Especial efforts were concentrated on the development of reliable implantable glucose sensors to close the loop for insulin delivery system - an artificial pancreas [2]. Miniaturization of the sensor to needle size is a common engineering approach to obtain a sensor suitable for subcutaneous implantation.

The main method for the construction of these glucose sensors is the use of enzyme electrodes [3]. This is because of the high substrate specificity and high activity of enzymes as biological catalysts. The enzyme usually used is Glucose Oxidase (GOD), and detection is performed according to the following overall reaction:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2$$

Amperometric glucose biosensors are usually based on measuring the increase in the anodic current due to H_2O_2 oxidation, or the decrease in the cathodic current due to O_2 reduction. The H_2O_2 electrode based glucose biosensors are advantageous because of their ease of fabrication and possibility of constructing them in small sizes even when conventional technology is used. Reports of the construction of glucose sensors utilizing the oxygen electrode have also been presented [4-6].

The general size of the needle-type glucose sensors ranges from 16 to 28 gauge (1.65 to 0.36 mm). A sensor usually consists of three layers, an inner permeable selective membrane (which may or may not be present), an enzyme layer containing the Glucose Oxidase, and an outer semi-permeable membrane. The sensor generally consists of a platinum working electrode (polarized as anode) and silver [7], stainless steel [8], Ag/AgCl [9], or platinum [10] as counter electrode, used as a reference in two electrode systems. A three electrode system with a Ag/AgCl wire reference electrode placed inside the needle body has also been reported [11].

Shichiri et al were the first to report success in miniaturizing a glucose sensor and introduced the needle enzyme electrode which had an outer diameter of 1mm [7]. Since then, there have been many reports on the development of needle sensors [8,10,12,13]. The response times range from 16 to 100 secs, and linear ranges were from 10 to 27 mM. *In vivo* performance of needle sensors has been evaluated by subcutaneous implantation in animals [9,14-17]. The lifetimes of the implanted sensors ranged from 4 to 14 days, with a delay of at least 5 minutes between subcutaneous and blood glucose levels. Subcutaneous implantation in humans has also been performed and sensor response showed high correlation with blood glucose levels [18,19]. A telemetry system has been proposed for monitoring and control of the insulin delivery system [20].

A new possible application of the needle sensors is the monitoring of the glucose level in the blood stream (intravascular sensors). Continuous online monitoring of glucose concentration appears of crucial importance in the management of trauma cases involving extensive bleeding - hemorrhage. Hemorrhagic shock generally consists of two phases, the compensatory phase and the decompensatory phase [21] . Hyperglycemia is associated with a deterioration of mechanisms which support homeostasis in the decompensatory phase of hemorrhagic shock [21,22]. Plasma glucose has been shown to follow a certain pattern during hemorrhagic shock. At the onset of the hemorrhage, the plasma glucose level rises to hyperglycemic levels from 90 mg/dL to 240 mg/dL in 2 hours.This is known as the decompensatory phase. This is followed by the compensatory phase in which plasma glucose levels start to fall, reaching hypoglycemic levels of approximately 30 mg/dL. It has also been demonstrated that early glucose administration to maintain the hyperglycemic phase arrests decompensation [23].

The compensatory phase of hemorrhagic shock lasts for approximately 2 hours [23], making the time delay and accuracy of subcutaneous monitoring of glucose levels unacceptable. Thus, hemorrhagic shock requires direct monitoring of glucose in the bloodstream for maximum accuracy and fastest response. Intravenous monitoring presents several complications such as risk of infection, vascular thrombosis, and inactivation of sensing devices by clotting.

In this communication we present initial results on the development of the needle sensor for monitoring of glucose levels during hemorrhagic shock.

EXPERIMENTAL

Reagents and Materials

Glucose Oxidase (GOD, E.C.1.1.3.4, activity 250 EU.mg^{-1}, purified from *Aspergillus Niger*) and Bovine Serum Albumin (BSA) were from Sigma Chemical Co.(St. Louis, MO), and used without further purification. The immobilization reagents: 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate and glutaraldehyde (25% aqueous solution) were also obtained from Sigma. Platinum wires of 0.127, 0.25 and 0.5 mm diameters were obtained from needles (outer diameter 2.11 mm and 1.27 mm) manufactured by Becton Dickinson Standard & Co. (Rutherford, NJ) were used. Silicone Water-Based Elastomer - Silastic (DC 3-5025) was obtained from Dow Corning Corp. (Midland, MI). The Nafion® perfluorinated ionomer was obtained from Aldrich as 5 wt% solution and used after dilution to 0.5% (w/v) using equal amounts of 2-propanol and distilled water. Ultrapure Low Temperature Isotropic (ULTI) carbon powder (fraction less than 325 mesh) was from Carbomedics. The standard polycarbonate membranes (pore size 0.03 μm, thickness 6 μm, and pore density 6 x 10^6 pores/cm^2 rated by the manufacturer) were from Poretics Corp. (Livermore, CA). D-glucose, anhydrous (Baker Analyzed, Phillipsburg, NJ) was used as a stock solution (20 g/L) in phosphate buffer, prepared at least one day before measurements for mutarotation. All other reagents used were of analytic grade and used without further purification.

Enzyme Immobilization

The enzyme Glucose Oxidase (GOD) was immobilized on ULTI carbon powder using the carbodiimide technique as described before [24]. Prior to sensor preparation, the enzyme-modified carbon powder was dispersed in 1 ml of 0.1M phosphate buffer solution (pH 7.4), containing fresh GOD (20 mg/ml), BSA (42.5 mg/ml) using an ultrasonic bath. In the preparation of the enzyme gel matrix, 2% glutaraldehyde was used for crosslinking the enzyme-modified carbon with BSA.

Membrane Coating

The standard polycarbonate (PC) membranes were used as obtained (without coating) or coated by Nafion or Silastic. For Nafion coating the samples of dish for one hour. They were then held vertically and dried in air for one hour. A disc (diameter c.a. 8 mm) was cut from the central area of the membrane sample and used in the sensor preparation as a glucose diffusion membrane.

The polycarbonate membrane was covered by a silastic layer to obtain the necessary diffusion properties. The preparation and optimization of this external glucose membrane has been the subject of our recent report [25].

Sensor Design

The stainless steel needle was cut at both ends to remove the plastic cap and the pointed end, and the ends were smoothed using files and fine sand paper. The needle was cleaned in concentrated nitric acid, washed with distilled water and dried. A platinum wire was cleaned in concentrated nitric acid and treated in a propane flame to form a smooth shape or a bulb at one of the ends. The platinum wire was insulated by dipping in silastic up to 2 mm below the bulb end, or by sealing in a polyethylene tubing with proper inner diameter and gluing with cyanoacrylate glue. Wires of 0.5 mm diameter were insulated using Teflon heat shrinkable tubing. The insulated platinum wire was then inserted into the body of the needle. A syringe was used to suck the immobilized enzyme gel (before final gelling) into the needle body using a plastic tube. A polycarbonate membrane (diameter c a. 8 mm) was used to cover the end of the sensor and was fixed in place using an o-ring. The sensor was then stored at 4 °C to accomplish the gelling process in the enzyme matrix. After that the polycarbonate membrane was carefully glued behind the o-ring with cyanoacrylate glue and then the o-ring was removed. The schematic of the assembled sensor is shown in Fig. 1.

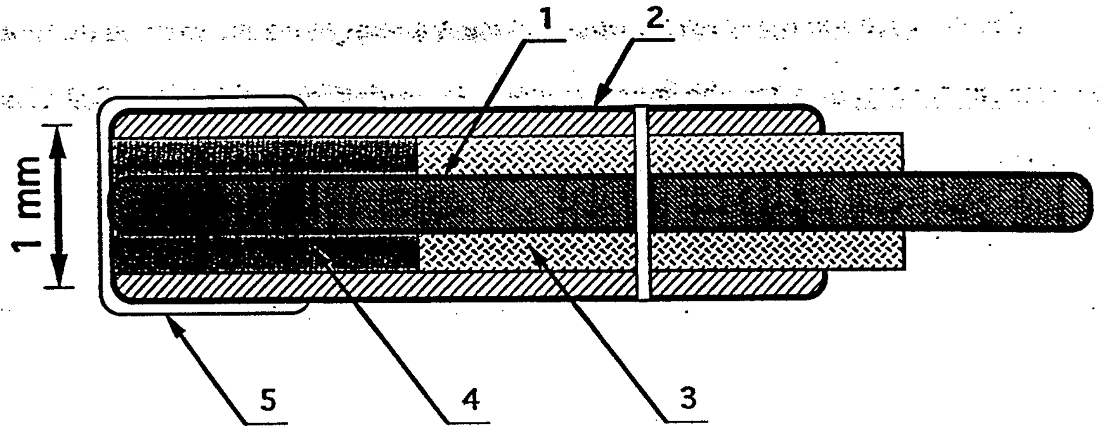


FIG. 1 Schematic cross-section of the glucose biosensor:
1. Platinum wire - working electrode (anode);
2. Stainless steel needle - counter electrode (cathode) and sensor body;
3. Insulation of the working electrode;
4. Enzyme gel matrix;
5. Polycarbonate glucose diffusion membrane;

Apparatus and Procedure

The needle sensors were tested *in vitro* in a magnetically stirred, thermostated measuring cell (volume 50 mL). A two electrode circuit was used, the platinum wire as a working electrode polarized as an anode, and the stainless steel needle body as both reference and counter electrodes, polarized as a cathode, and maintained by a potentiostat (BAS CV-1B, Bioanalytical Systems Inc., West Lafayette, IN). The amperometric signals were recorded on Omnigraphic 100 recorders (Houston Instruments, Austin, TX). The supporting electrolyte was 0.1M phosphate buffer solution, pH 7.4 containing 0.1 M KCl. Between measurements the sensors were stored soaked in phosphate buffer (pH 7.4) in closed vessels at 4 °C. Before measurements the sensors were polarized for at least 10 minutes to establish the background current.

The polarization curves were obtained by manually varying the potential of the platinum electrode versus the stainless steel electrode from +0.3 V to +0.9 V in 0.05 V steps and recording the steady-state value of the anodic current.

Calibration curves - the dependence of the steady state anodic current on glucose concentration - were obtained when the glucose concentration was varied by consecutive additions of glucose from stock solution to the measuring cell in 40 mg/dL concentration increments. Calibration curves in serum were obtained using serum samples prepared from bovine blood by centrifugal separation. Glucose levels in these samples are measured by routine clinical methods (Beckmann Glucose Analyzer) and then varied by addition of glucose from the stock solution.

Reproducibility of the sensor response was tested by recording the current signal to alternatingly varying glucose concentrations between 50 mg/dL to 100 mg/dL, and between 100 mg/dL to 200 mg/dL.

Hemorrhagic shock is simulated *in vitro* by varying the concentration of glucose in phosphate buffer solution by step changes from 100 mg/dL to 300 mg/dL and then, after 20 minutes from 300 mg/dL to 30 mg/dL. During these experiments the sensor amperometric transient response was continuously recorded.

RESULTS and DISCUSSION

Steady-state polarization curves were obtained in order to estimate a working potential for the prepared needle sensors. Fig. 2a shows a typical polarization curve of a needle sensor in the presence (100 mg/dL, curve 1) and the absence (curve 2) of glucose in the cell. In glucose solutions a significant increase of the anodic current at potentials more positive than +0.35 V occurs. The anodic

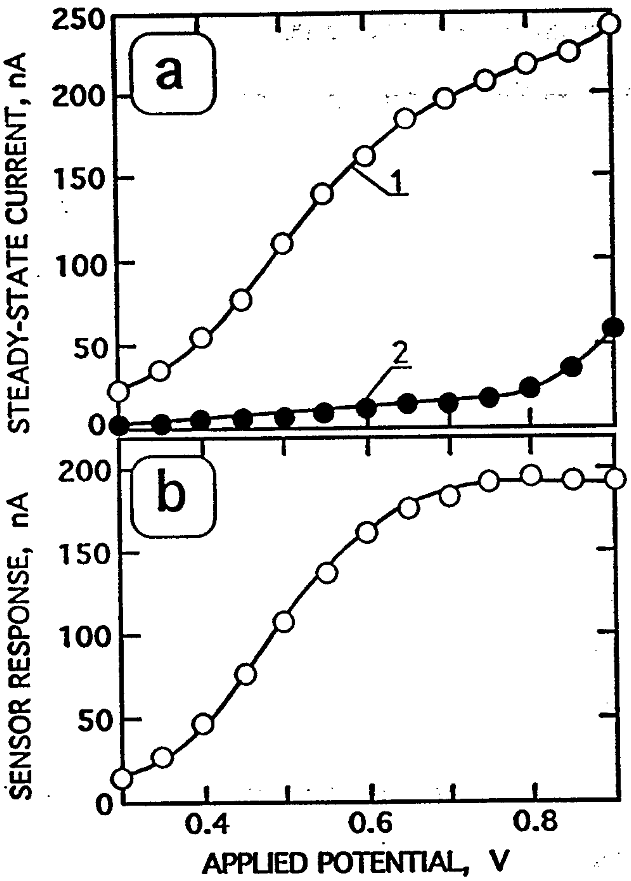


FIG. 2 Steady-state polarization curves of the platinum electrode vs stainless steel needle in the biosensor with standard polycarbonate membrane:

(a) in 320 mg/dL glucose solution (curve 1) and the background curve in blank phosphate buffer (curve 2);

(b) resulting polarization curve of the sensor response (subtraction of curve 2 from curve 1)

current did not reach an observable plateau due to the increase of the background current at higher potentials. The difference between the anodic current in glucose solutions and the background current (sensor response) is plotted against the applied potential (shown in Fig. 2b). It is clear that a plateau is reached at potentials more anodic than +0.65 V, the range of diffusionaly limited electrooxidation of hydrogen peroxide. A working potential of +0.7 V was chosen, and all sensors were operated polarized at this potential.

Sensors Response to Glucose

Calibration curves of the needle sensors with polycarbonate membranes (standard and treated with Nafion or Silastic) are shown in Fig. 3a. A linear dependence of the sensor response on glucose concentration can be observed for all three cases. The linear range (the concentration range within the calibration curve is linear) is affected by treatment of the membrane. In the case of standard polycarbonate membranes (curve 1) a linear dependence of the sensor response versus glucose concentration is estimated to be up to 240 mg/dL with sensitivity of 0.55 nA/mg/dL. Coating of the polycarbonate membrane with Nafion ionomer increases the linear range up to 400 mg/dL, simultaneous decreasing the sensor sensitivity to 0.47 nA/mg/dL (curve 2). A similar effect is also observed when membranes coated with Silastic are used (curve 3). The linear range is extended up to at least 500 mg/dL and the sensitivity is 0.36 nA/mg/dL.

The Eadie-Hofstee plot of the same data (Fig. 3b) can be used as a tool in diagnosis of diffusional limitations [26]. The external diffusional limitations provided by the standard polycarbonate membrane affect the Eadie-Hofstee plot and the curve becomes concave towards the origin of the axes (curve 1). A portion of this curve corresponding to a kinetically controlled mode of the sensor operation can be located from which an apparent Michaelis-Menten constant is obtained as 600 mg/dL (39.2 mM). When coated membranes are used (curve 2 - Nafion coated PC membrane and curve 3 - PC membrane coated by Silastic) the external diffusion limitations are totally dominant and the curves become asymptotic to the current

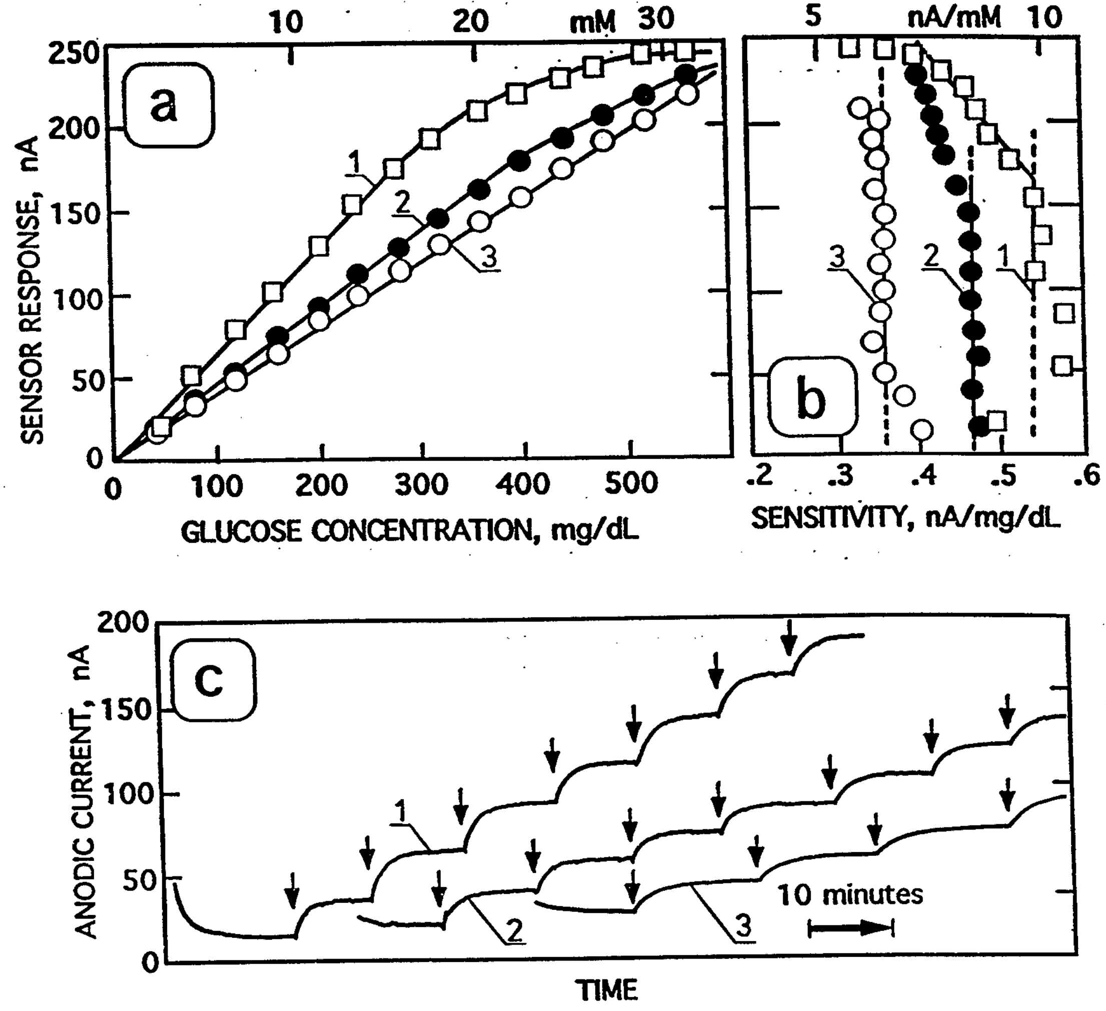


FIG. 3 Dependence of the biosensor amperometric response on glucose concentration:
(a) calibration curves of the biosensor in glucose buffer solution:
(b) Eadie-Hofstee plot of the data from (a);
(c) protocols of the biosensors response to consecutive additions of glucose (moments of the glucose addition are shown by arrows).
Curves:
1 biosensor with standard (uncoated) polycarbonate membrane;
2 biosensor with polycarbonate membrane treated by Nafion;
3 biosensor with Silactic coated polycarbonate membrane.

signal (Y) axis. A tendency to depart from this vertical line (constant sensitivity) can be seen at high current responses (which correspond to high glucose concentrations).The diffusion limited operation of the sensor response is also confirmed in the calibration tests performed with different agitations in the measuring cell - there is no difference in the sensor response when the stirring speed is varied from 100 to about 600 r.p.m..

The response time (estimated as time to reach 95% of the steady-state value of the current signal) is also affected by use of the coatings. Fig. 3c presents typical protocols of the current transients for the three sensors during the calibration test. It can be seen that the response time of each membrane does not depend on concentration when sensor operates within its linear range. The response time in this case is a function of the concentration step-change only. The response time (for a concentration step-change of 40 mg/dL) for the sensor with standard PC membrane is 3 to 4 minutes; for the sensor with Nafion treated membrane it is 4 to 6 minutes and for the sensor with membrane coated by Silastic, 7 to 9 minutes. At the highest glucose concentrations, when kinetics become rate limiting factor, the response time increases with increasing concentration of glucose.

The reproducibility of the sensors response is tested by varying the glucose concentration in the measuring cell alternately between two levels with concentration step-changes of 50 mg/dL: from 50 mg/dL to 100 mg/dL (2.8 - 5.6 mM), and with step changes of 100 mg/dL: between 100 mg/dL and 200 mg/dL (5.6 - 11.1 mM). The sensors monitored the glucose level in the cell for two hours between concentration changes. Continuous testing for more than five days was performed; overnight sensors were left operating at lowest glucose concentrations. It is found that for every level of glucose concentration, the sensor response attains the same amperometric signal value within an acceptable ±5% error limit. The response time at the concentration step-change of 100 mg/dL is 10 to 12 minutes for the sensors with standard and Nafion treated membranes and about 15 minutes for the sensor with a membrane coated by Silastic. There is no observable difference between the response times for increasing concentration, and for decreasing concentration.

The glucose concentration profile during hemorrhagic shock is simulated by a concentration step change from 100 mg/dL (approximating the normal physiological value) to 300 mg/dL, and after 30 minutes (compensatory phase) a decreasing step change from 300 mg/dL to 30 mg/dL (decompensatory phase). Typical transients of the current sensors responses to this test are shown in Fig. 4. The sensor with standard PC membrane (curve 1) reaches the steady-state value in 16 minutes during the increasing step and in 20 minutes during the decreasing step of the test. For the sensor with Nafion treated PC membrane (curve 2) the response times are 16 minutes for the increasing and 22 minutes for the decreasing step. Sensors with Silastic coated PC membrane responded in 25 minutes to increasing glucose concentration, and in 35 minutes to decreasing concentration to the hypoglycemic level. These tests have been performed several times during continuous operation of the sensors for a week. The values of the steady-state amperometric response of this sensor have been found to be within 10% of values predicted from the calibration curves.

It should be noted that the concentration change during these tests is a step, as opposed to the gradual concentration changes in the physiological processes. These results show that the sensors are able to respond to the varying glucose concentrations in an acceptable time and with reproducible current values. It can be

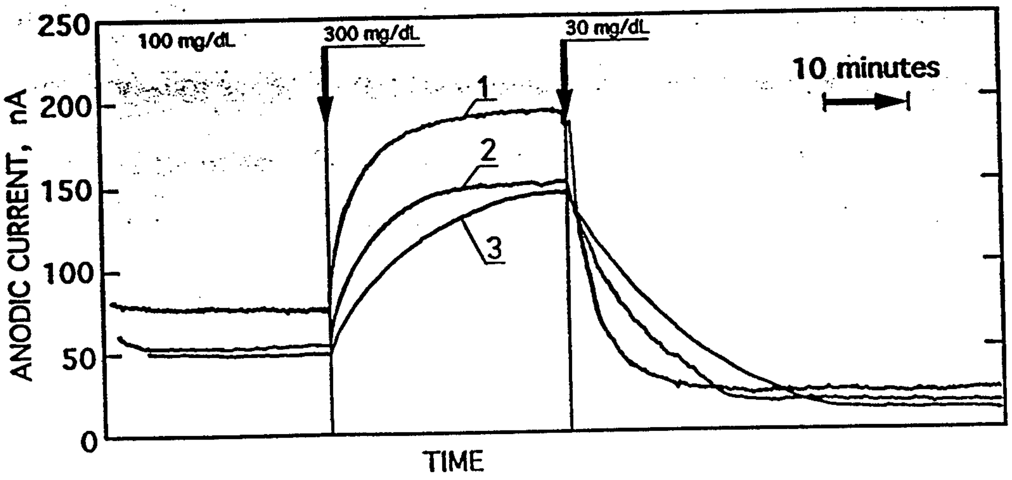


FIG. 4 Protocol of the sensors response during hemorrhage *in vitro* simulation test (moments of glucose concentration change are shown by arrows).
Curves:
1 biosensor with standard (uncoated) polycarbonate membrane;
2 biosensor with polycarbonate membrane treated by Nafion;
3 biosensor with Silactic coated polycarbonate membrane.

seen that the use of Silastic coatings on polycarbonate membranes has the advantage of increasing the linear range of the sensor response, but with the disadvantage of increasing the response time.

The evaluation of the sensor performance in serum can be used as an initial interference test. Calibration curve in bovine serum is obtained by procedure similar to that when phosphate buffer solution is used. Fig. 5 presents a comparison between the calibration curve in bovine serum and two calibration curves in phosphate buffer solution (obtained before and after the serum test) for a sensor with standard PC membrane. The calibration curve in bovine serum starts from concentration of glucose 85 mg/dL (the actual concentration of the probe) to avoid dilution. The calibration curves in buffer solution are presented without subtraction of the background current so as to be comparable with the curve obtained in sera. It

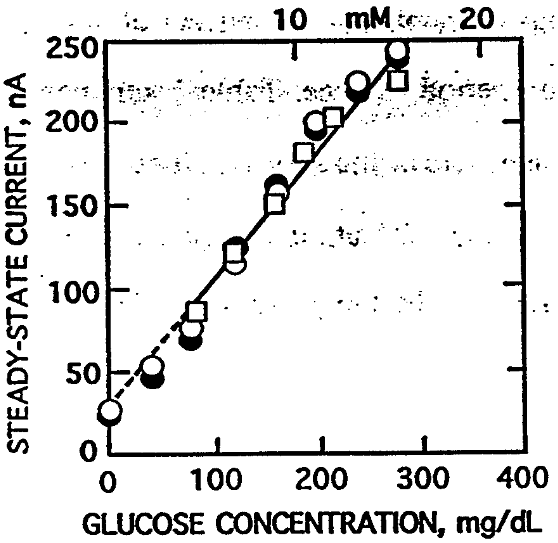


FIG. 5 Calibration curve of glucose biosensor with standard polycarbonate membrane in bovine serum (□) compared with the calibration data obtained before (○) and after (●) serum test.

can be seen from the Figure that the extrapolated background current of the calibration curve in serum and its slope are practically the same as those of the calibration curves obtained in phosphate buffer. Similar coincidences are obtained with sensors employing Nafion treated and Silastic coated membranes.

Sensor Life Times

The stability of the sensors in time is recognized as one of the most important factors with respect of practical applications. Two aspects of the sensors life-time have been studied: the storage life-time and the long-term operational stability.

In order to estimate the storage life-time, three sets of sensors with standard PC membrane, with PC membrane treated by Nafion, and with Silastic coated PC membrane (six sensors in each series) were made. These sensors were then stored in closed vessels (soaked in phosphate buffer) at 4°C and calibration curves obtained after a certain period of time. Table 1 summarizes the data of this test presenting the main parameters of the sensor calibration curves: the sensitivity (the slope in the linear part of the calibration curve) and the linear range of the sensor response to glucose (presented by the upper limit of the linear range). It should be mentioned that the presented data are from the first measurements for each of the sensors in the set. Storage time is counted from the day of sensor preparation. It can be seen that in each of the sets, sensors demonstrate typical values of their sensitivity and linear range: relatively higher sensitivity and lower linear range for the sensor with standard PC membranes with respect to the sensors with coated membranes. Within each series the difference in the parameters of the calibration curves do not exceed a variation of ±10%. These sets of sensors were tested for a period of 8 weeks (56 days) and no practical change of their performance (within ±10%) has been seen. To obtain the actual limits of the storage life-time of the sensors another three sets have been prepared and the tests are currently in progress.

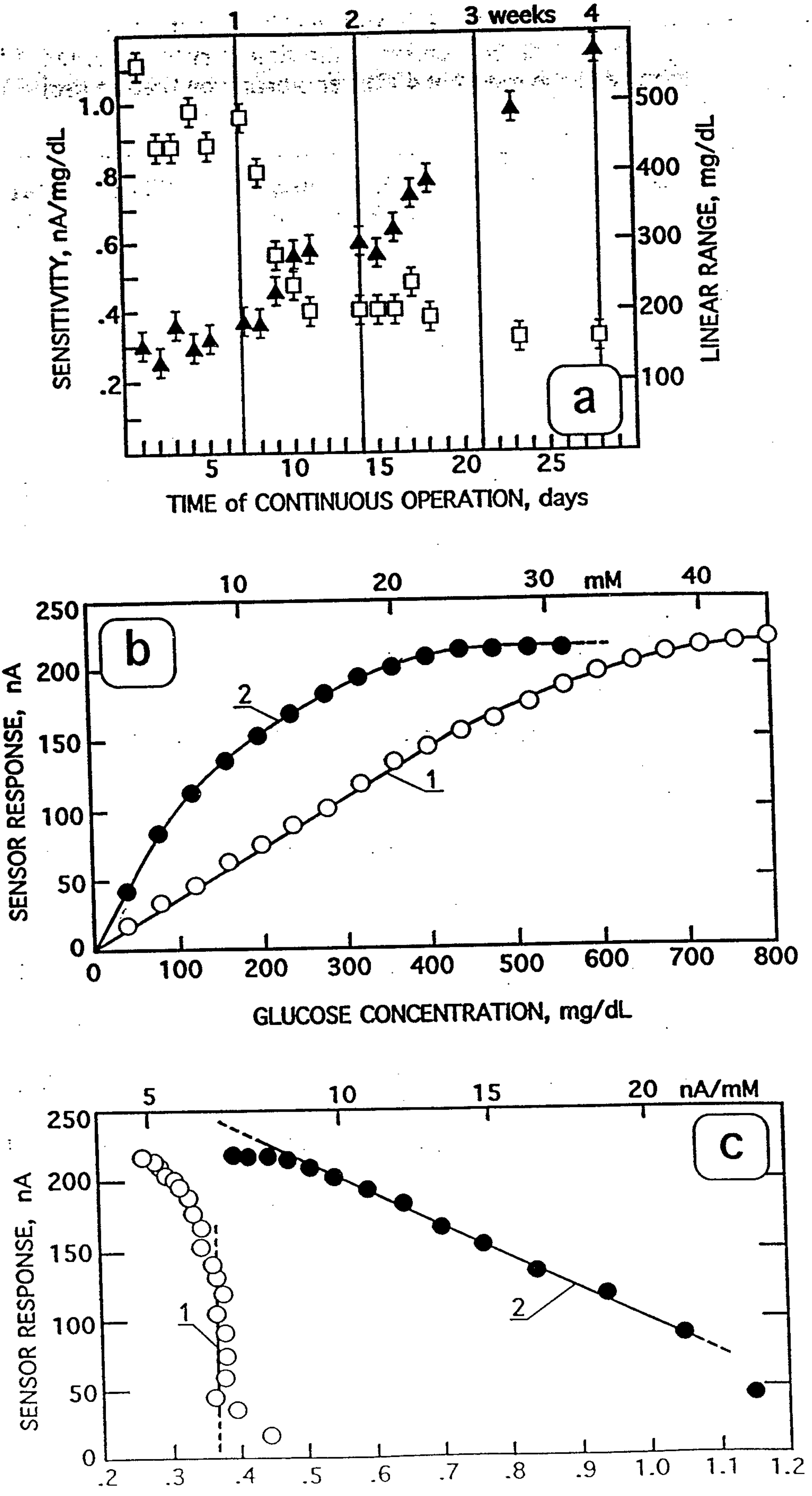
The operational long-term stability of the sensor can be defined as the period of time over which the sensor can continuously monitor glucose concentration. During the long-term operational stability tests sensors were kept operating in glucose solution with concentration of 100 mg/dL and calibration curves of the sensors were periodically obtained. Fig. 6a presents the evaluation of the parameters of the calibration curves (sensitivity and linear range) for a sensor with PC membrane coated with Silastic. The plot shows that the sensitivity and the linear range remained practically constant (within ±10%) for first week of

TABLE 1. Storage Long-term Stability of the Needle Sensors
Parameters of the sensor calibration curves, obtained after storage of the sensor at 4°C after preparation (before use)

Storage Time	Standard PC membrane		Nafion coated PC membrane		Silastic coated PC membrane	
days	Sensitivity nA/mg/mL	Linear Range mg/dL	Sensitivity nA/mg/mL	Linear Range mg/dL	Sensitivity nA/mg/mL	Linear Range mg/dL
1	0.55	240	0.47	400	0.36	500
7	0.51	280	0.45	440	0.41	580
14	0.58	240	0.41	440	0.42	540
28	0.54	220	0.50	420	0.38	540
35	0.48	240	0.43	440	0.36	500
56	0.56	280	0.43	400	0.35	540

continuous operation. After that the sensitivity of the sensor increases, among with a decrease of the linear range.

Fig. 6b shows two calibration curves for this sensor: obtained after 7 days (curve 1) and after 28 days of sensor continuous operation. A marked decrease in the linear range and an increase in sensitivity can be seen in this figure together with a change of the shape of the curve. The corresponding Eadie-Hofstee plots of the data presented in Fig. 6b are shown in Fig. 6c. The Eadie-Hofstee plot of the sensor calibration curve after one week of operation (curve 1) is close to that obtained at the first measurement (compare with Fig. 3b, curve 3). The diffusion limitations of the sensor response can be demonstrated by the well defined zone of constant sensitivity - the plot is a line asymptotic to the current response axis. The Eadie-Hofstee plot obtained from the calibration curve after 4 weeks operation


1
2
3 weeks
4
a
SENSITIVITY, nA/mg/dL
1.0
.8
.6
.4
.2
LINEAR RANGE, mg/dL
500
400
300
200
100
5
10
15
20
25
TIME of CONTINUOUS OPERATION, days
10
20
30 mM
40
b
SENSOR RESPONSE, nA
250
200
150
100
50
0
2
1
0
100
200
300
400
500
600
700
800
GLUCOSE CONCENTRATION, mg/dL
5
10
15
20 nA/mM
c
SENSOR RESPONSE, nA
250
200
150
100
50
0
1
2
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2

(curve 2) is a line with negative slope and it shows that the sensor is operating under kinetic rate control. The apparent Michaelis-Menten constant calculated from the slope of curve 2 is 300 mg/dL (16.8 mM).

Two possible explanations of such behaviour can be offered: loss of the diffusion properties of the membrane, or structural changes in the enzyme gel matrix. Optical microscopy observations do not show any noticeable damage of the membrane. Loss of the Silastic coating can be supposed, but the calibration curve parameters in this case should become close to that of the sensor with standard PC membrane (compare Fig. 3a and b, curves 1 with Fig. 6b and c, curves 2). The sensor with PC membrane coated by Silastic after 4 weeks of continuous operation shows a lower linear range and higher sensitivity than the freshly prepared sensor with uncoated membrane. The apparent Michaelis-Menten constant is two times less than that obtained with the sensor with the standard (uncoated) membrane. This lead us to attach significance to the second hypothesis - changes in the gel with the immobilized enzyme. Bearing in mind that during inactive storage no evidence of such behavior is observed (Table 1), it can be assumed that possible destruction of the gel is caused by hydrogen peroxide evolved in the enzymatic reaction. In its diffusion to the platinum electrode it may form micro-channels in the gel matrix, changing its properties. This may cause a change in the diffusion conditions in the gel matrix and to be a possible explanation for the sensor behaviour during the long-term operational stability test.

FIG. 6 Long-term operation stability of the biosensor:
(a) dependence of the biosensor sensitivity (▲) and the linear range (☐) on the time of continuous operation in 100 mg/dL glucose solution;
(b) calibration curves of the biosensor obtained after 7 (curve 1) and after 28 days of continuous operation;
(c) Eadie-Hofstee plot of the data from (b).

CONCLUSIONS and FUTURE WORK

This communication presents initial results on the development of a needle glucose biosensor for monitoring of glucose levels during the hemorrhagic shock. The sensor employs commercially available and well defined polycarbonate membranes as diffusion membranes for glucose. The use of Nafion and Silastic coated membranes extended the linear range up to 440 mg/dL and 540 mg/dL respectively, and also a corresponding increase in the response time. The sensor responded adequately to an *in vitro* simulation of hemorrhagic shock closely following the protocol. Long term stability studies of the sensors show that they do not change their main characteristics when stored for at least 8 weeks or operated continuously for 7 days. Experiments on the long-term stability of the sensors are still in progress. Further work will include also detailed interference tests as well as sensor evaluation in whole blood *in vitro*. Glucose level monitoring during hemorrhage shock will be performed with these sensors when implanted intravenously in laboratory animals (rats).

ACKNOWLEDGEMENTS

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Needle-Type Glucose Biosensor with an Electrochemically Codeposited Enzyme in a Platinum Black Matrix

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APPENDIX 2

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Abstract
In this study we report the development of a needle-type glucose biosensor for the management of hemorrhagic shock. The immobilization technique depends on the electrophoretic deposition of the glucose oxidase enzyme in an electrochemically grown platinum black matrix. The sensor was coated with Nafion to decrease the effect of interferents. Preliminary tests were carried out to evaluate the sensor performance in vitro. These tests included the measurement of glucose levels in buffer solutions containing various potential physiological interferents, as well as in bovine serum. The preliminary results show the sensor to have linearity up to 33 mM and a diminished response to interferents. The advantages of this technique are its simplicity and high controllability.

Keywords: Electrodeposited enzyme, Needle glucose biosensor

1. Introduction

The development of sensors for invasive glucose monitoring has been the focus of much research during the past decade [1]. This attention arises from the clinical importance of these sensors, especially in the management of diabetes [2] and hemorrhagic shock [3]. Amperometrix enzyme electrodes present the most common approach because of the high substrate specificity and activity of the enzymes, and the proportionality of the current signal to the glucose concentration provided by amperometry. The oxidation of glucose by molecular oxygen to form gluconic acid and hydrogen peroxide, which is catalyzed by glucose oxidase (GOD), presents the basis for glucose detection.

Needle-type glucose sensors based on the hydrogen peroxide electrode have been the most attractive because of their ease of fabrication and adequacy for implantation [4–6]. Success with this approach has reached the stage of implantation and calibration experiments [7, 8]. In the fabrication of these sensors, glucose oxidase can be immobilized by different methods, such as cross-linking with glutaraldehyde and bovine serum albumin, or by entrapment in conducting or nonconducting polymers [9], or by immobilization on carbon particles [10]. One method of fabrication which has not been investigated completely is the codeposition of GOD and metal particles on the surface of transducer electrodes. Ikariyama and co-authors were the first to propose such a technique using platinum as an entrapping matrix for GOD [11, 12]. This method makes use of two electrochemical techniques that can be performed simultaneously. Namely, the electrolytic reduction of metal complexes and the electrochemical adsorption of proteins. The deposited metal particles act as both a transducer and a matrix for enzyme immobilization. This technique has been used for the fabrication of microenzyme sensors for flow-injection analysis [13, 14]. Abe et al. [15] used a similar technique for the preparation of microsensors for the intracellular monitoring of glucose levels. The work of these researchers demonstrated the feasibility of this technique for the fabrication of micro-biosensors.

In this article, we use a technique similar to that proposed by Ikariyama et al. [11] for the fabrication of needle-type sensors. The ultimate goal in developing these sensors is the monitoring of glucose levels during hemorrhagic shock. This application requires a diminished response to common physiological interferents and linearity up to at least 20 mM. Nafion coatings have been employed in order to accomplish this.

2. Experimental

2.1. Reagents and Materials

The glucose oxidase (GOD, E.C.1.1.3.4, activity 250 $\mathrm{EU.mg^{-1}}$, from *Aspergillus niger*) was from the Sigma Chemical Co. (St. Louis, MO), and used without further purification. The sodium hexachloroplatinate(IV) hexahydrate and lead(II) acetate trihydrate were from the Aldrich Chemical Co., Inc. (Milwaukee, WI), and used as obtained. Platinum wires of 0.127 mm diameter were obtained from the Aldrich Chemical Co., Inc. (Milwaukee, WI). Stainless steel 18 gauge needles (outer diameter 1.27 mm) manufactured by Becton Dickinson Standard & Co. (Rutherford, NJ) were used. The Nafion perfluorinated ionomer was obtained from Aldrich as 5 wt.% solution and used after dilution to 0.5% using equal amounts of 2-propanol and distilled water. Anhydrous D-glucose (Baker Analyzed, Phillipsburg, NJ) was used as a stock solution (20 g/L) in phosphate buffer, prepared at least one day before measurements for mutarotation. The interferents used: 4-acetamidophenol (acetaminophen; APAP; *N*-acetyl-*p*-aminophenol), uric acid (2,6,8-trioxypurine), L-ascorbic acid, and glycine were from the Sigma Chemical Co. (St. Louis, MO). Urea was obtained from Fisher Scientific Co. (Fair Lawn, NJ). All other reagents used were of analytical grade.

2.2. Sensor Preparation

The stainless steel needle was cut at both ends to remove the plastic cap and the pointed end, and the ends were smoothed using files and fine sandpaper. The needle was cleaned in concentrated nitric acid, washed with distilled water, and dried. A platinum wire was cleaned in concentrated nitric acid and treated in a propane flame to form a smooth shape or a bulb at one of the ends. The platinum wire was insulated by dipping in Silastic up to 2 mm below the bulb end.

The platinization and electrophoretic incorporation of the enzyme particles was carried out in a solution containing 33 mg

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3.1. Sensor's Response to Glucose

Calibration plots of the needle sensors with GOD immobilized by entrapment in a platinum black matrix are shown in Figure 3a. A nonlinear dependence of the steady-state current on the glucose concentration is observed. The sensor signal tends to saturation at a glucose concentration greater than 60 mM. A linear dependence of the sensor response on the glucose concentration is observed when the enzyme electrode is coated with Nafion (curve 2). The sensor signal is linearly proportional to the substrate concentration up to 33 mM glucose with a sensitivity (slope of the linear portion) of 0.036 μA/mM. The signal then tends to a plateau with a saturated signal value approximately three times less than that of the uncoated sensor.

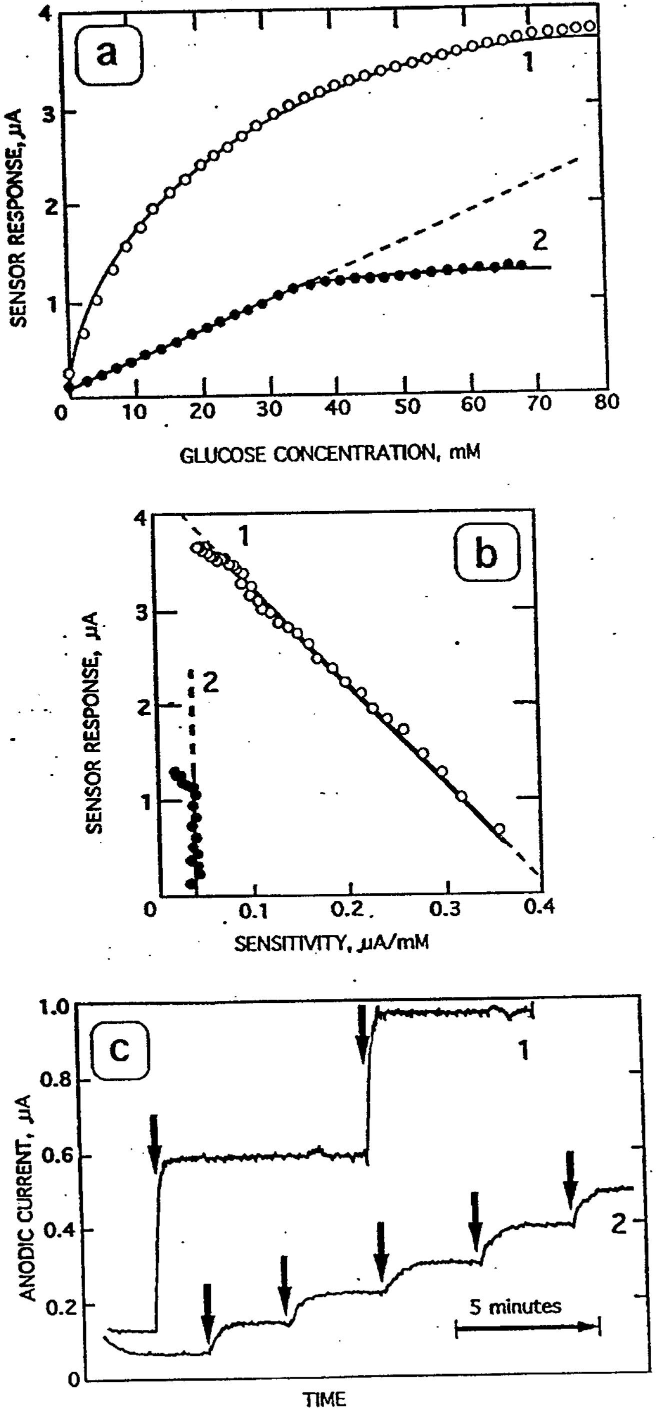

Fig. 3. Dependence of the biosensor amperometric response on the glucose concentration for uncoated (curves 1) and Nafion coated electrodes (curves 2): a) calibration plots of the biosensor in glucose buffer solution; b) Eadie–Hofstee plot of the data from a); and c) protocols of the biosensors response to consecutive additions of glucose (the points at which the glucose is added are shown by arrows).

Eadie–Hofstee plots of the data from the calibration plots (Fig. 3b) can be used as a tool in the diagnosis of diffusional limitations. The uncoated sensor shows a typical linear dependence using the Eadie–Hofstee coordinates (curve 1) corresponding to the Michaelis–Menten kinetics of the enzymatic reaction, which are the rate limiting factor in this case

$$I[\mu A] = 4.28 \pm 0.02[\mu A] - 12.26 \pm 0.04[\mathrm{mM}] \times I/C[\mu A/\mathrm{mM}] \quad (1)$$

The external diffusional limitations provided by the Nafion layer affect the Eadie–Hofstee plot and the curve becomes a vertical line parallel to the current axis (curve 2). The diffusion-limited operation of the sensor response is also confirmed in the calibration tests performed with different agitations in the measuring cell – there is no difference in the sensor response when the stirring speed is varied from 100 to about 600 rpm.

The response time (estimated as the time to reach 95 % of the steady-state value of the current signal) is also affected by using a Nafion coating. Figure 3c presents typical protocols of the current transients for the two sensors during the calibration test. It can be seen that the response time of each does not depend on the concentration, being a function of the concentration step-change only. The response time (for a concentration step-change of 2 mM) for the sensor without a coating is less than 20 s; and for the sensor with a Nafion coating it is 1 min. At the highest glucose concentrations, for the sensor without a coating, the response time increases with increasing concentration of glucose, while for the Nafion coated sensors it remains independent of the concentration.

It should be noted that the concentration of the glucose during these tests changes stepwise, as opposed to the gradual concentration changes in the physiological processes. The results show that the sensors are able to respond to the varying glucose concentrations with an acceptable response time (approximately 1 min). It can be seen that the use of Nafion coatings on the electrode with GOD entrapped in a platinum black matrix has the advantage of increasing the linearity of the sensor response over the concentration range of physiological interest, with an increase of the sensor response time, that is still acceptable from a practical point of view. Sensors prepared using this technique show reproducible current values (sensitivity), a linear range, and a response time within 10 %.

Table 1 shows the effect of additions of carbohydrates and other common interferents. Examining the data for the uncoated sensor, it can be seen that the response to carbohydrates (other than glucose) is negligible with respect to the sensor response to the same concentration of glucose. This indicates that the sensor signal is due to the oxidation of glucose by GOD and not the electrochemical oxidation of glucose on the platinum electrode surface. The fact that the sensor response to these carbohydrates is above zero may be explained by the activity of the platinized platinum electrode during the reaction of direct electrooxidation of the carbohydrates [17]. Due to this activity, the uncoated sensor demonstrates a significant amperometric response to common interferents. For example, the signal due to direct electrooxidation of uric acid (0.33 mM) is 2.1 times higher than the response to 4.4 mM glucose (concentration within the normoglyceamic range). Nafion coated sensors, on the other hand,

Table 1. Response of uncoated and Nafion-coated sensors to carbohydrates and other common interferents.

Substance	*Concentration [mM]*	*Sensor response*	
		Uncoated [nA]	*Nafion-coated [nA]*
Glucose	4.4	465	105
Sucrose	4.4	8	<1
Fructose	4.4	6	<1
Lactose	4.4	2	<1
Glycine	0.5	35	21
Urea	8.5	25	17
Ascorbic acid	0.125	28	14
Acetaminophen	0.13	420	172
Uric acid	0.33	981	352

Table 2. Sensor response to glucose in buffer solution and in bovine serum.

Glucose concentration [mM]	*Sensor response in:*		
	Buffer solution before serum test [nA]	*Bovine serum [nA]*	*Buffer solution after serum test [nA]*
4.4	63	92	104
6.6	94	122	127
8.4	137	157	155
10.2	186	187	188
12.1	201	205	205
15.6	230	213	223

eliminated the sensor response to the other tested carbohydrates (sucrose, fructose, and lactose). The sensor signal in response to other common interferents was also reduced by at least 50 %.

Table 2 shows the evaluation of the sensor performance in serum and in buffer solution before and after the serum test. Comparing the sensor response in buffer solution before the serum test with that during the serum test, it can be seen that the values of the sensor response to a glucose concentration from 10 to 12 mM in both measurements coincide (within 2 %). At higher glucose concentrations, the signal in serum is lower than that obtained in buffer solution, but with an acceptable deviation of 7 %. A higher response of the sensor is observed for glucose levels in serum within the range 4.4–8.4 mM. The values of the sensor response obtained in buffer after the serum test practically coincide with those obtained in serum.

4. Conclusions

A needle-type glucose biosensor has been developed. Fabrication of the sensor is based on the deposition of GOD in a platinum black matrix. The sensor has a large linear range and a fast response. A Nafion coating diminished the sensor response to common interferents by at least 50 %. The sensors were operated for several weeks, being stored at 4°C between measurements. They operated well in serum, showing close correlation with the response in buffer solution.

This article presents the initial in vitro evaluation of the sensor performance. Experiments for the evaluation of the sensor performance in whole blood are currently in progress. Future in vivo experiments will focus on the intravenous monitoring of blood glucose levels during intentionally initiated hemorrhagic shock in rats.

5. Acknowledgement

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APPENDIX 3

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Analytica Chimica Acta 313 (1995) 45–54

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Development of a needle-type biosensor for intravascular glucose monitoring ☆

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Abstract

A needle type glucose biosensor has been developed for monitoring glucose levels during the hemorrhagic shock in trauma patients. The sensor employs polyurethane, cellulose acetate or PVC layers as an outer glucose diffusion and protective membrane, and glucose oxidase enzyme entrapped in poly(1,3-phenylenediamine) film. Sensor performance was evaluated in vitro and the sensor shows a sensitivity of up to 35 nA/mM and a linear range of up to 38 mM. Evaluation of the sensor response in serum showed similar sensitivity and linear range as obtained using calibration curves in buffer solution. The sensor has a short response time of 23 s. The sensors were operated continuously for 14 days in phosphate buffer solution, and no significant change in the sensitivity and the linear range was observed during the first 5 days. Sensors show a minimum change in their performance when stored inactive in buffer at 4°C for at least eight weeks.

Keywords: Biosensors; Glucose; Phenylenediamine; Polymer coatings

1. Introduction

The importance of biosensors has increased considerably during the past decade, as they combine the specificity of biological systems with the advantages of electrochemical transduction [1]. Glucose biosensors have received much attention because of their many applications in clinical chemistry, biotechnology and the food industry. The research efforts have in particular been directed towards the development of an amperometric sensor for glucose, due to the importance of glucose for diabetic patients [2]. Success in this research has reached the level of short term human implantation [3]. Another possible application of glucose sensors is in the management of hemorrhagic shock [4]. The importance here lies in increasing the survival rate of injured patients at risk of hemorrhagic shock, while they are being transported to a hospital. Unlike diabetes, hemorrhagic shock requires direct intravascular implantation of these sensors, and the monitoring of glucose levels for short periods of time (up to several hours).

An important step in the fabrication of glucose biosensors is the immobilization of glucose oxidase (GOD) enzyme, and various methods have been used for this process. These methods include cross-linking with glutaraldehyde, chemical immobilization on carbon carriers [5], entrapment in polymer layers [6,7], in metal matrices [8,9] or electrochemically grown polymer films on the electrode surface [10].

☆ Invited paper on Analytical Biotechnology.

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Electropolymerization is the electrochemical oxidation of a monomer from a solution to form a conducting or non-conducting polymer on the electrode surface. Enzymes can be used in the solution and incorporated into the polymer. This approach has several advantages. First, this process can be controlled by the electrode potential, and therefore allows control of the polymer film thickness and hence the amount of entrapped enzyme. Second, since the polymerization occurs locally at the electrode surface, it can be used to confine an enzyme precisely at the electrode without cross-immobilizing it on a neighboring electrode. This property is suitable for the fabrication of micro-arrays. Third, it is possible to use this technique to build multilayer structures, either one or more enzymes layered within a single polymer, or one enzyme within a multilayered copolymer [10].

Foulds and Lowe [11] and Umana and Waller [12] were the first to demonstrate the possibility of employing GOD entrapped in poly(pyrrole) in the fabrication of amperometric glucose sensors. Since then, much research effort has been directed towards the development of glucose sensors employing this concept [13]. Other approaches in fabrication of pyrrole sensors besides physical entrapment of GOD have been employed. For example, covalent electropolymerization of GOD to pyrrole by modifying different side chains on the enzyme has been reported [14]. The modification reactions involved carbodiimide coupling or Schiff base formation [15]. Foulds and Lowe demonstrated the possible use of covalently attached ferrocenes to pyrrole monomers as a step towards the fabrication of "reagentless" mediated sensors [16]. There have been other reports on incorporating mediators in pyrrole films as anionic counter ions [17,18].

The fact that some conducting polymer films become non-conducting in the presence of enzymatically produced H_2O_2 has prompted investigators to use non-conducting polymers to immobilize enzymes [10]. First, the polymer growth is self limiting, thus producing thin polymer films resulting in a short sensor response time. The thin film also allows a higher enzyme content leading to larger sensor response and a high sensitivity. It has been reported that non-conducting polymers also have a characteristic permselectivity that greatly decreases the effect of common physiological interferents on the sensor response [19]. The literature includes reports on the use of non-conducting polymers, such as poly(phenol) [20–22] and poly(indole) [23], and poly (phenylenediamines) [24,25,19,26–29] in the fabrication of glucose biosensors.

A non-conducting polymer that has been the focus of much research is 1,2-phenylenediamine. Yacynych et al. [24] were the first to demonstrate that the oxidation of 1,2-phenylenediamine was irreversible, resulting in the formation of an insulating polymer film completely covering the electrode surface. After the concept of entrapping GOD in these non-conducting polymer films had been demonstrated, numerous studies have appeared applying this in the fabrication of glucose sensors [19,26–29].

Sasso et al. [19] proposed a glucose sensor having a platinized reticulated vitreous carbon electrode with GOD entrapped in a poly(1,2-phenylenediamine) film. Malitesta et al. [26] constructed a glucose sensor, employing the same principal, but using platinum electrodes. Wang et al used a similar construction but with an additional outer lipid layer to provide diminished sensor response to acetaminophen (a common physiological interferant) [27].

Poly(1,3-phenylenediamine) has also been employed in the construction of glucose sensors. Geise et al. [28] have demonstrated the use of 1,1′-dimethylferrocene as a mediator entrapped with GOD in a poly(1,3-phenylenediamine) film. Yacynych et al. [29] have also used poly(1,3-phenylenediamine) for the fabrication of miniaturized glucose sensors.

In this paper, we have employed 1,3-phenylendiamine in the development of a needle-type glucose sensor. The ultimate goal in the development of this sensor is to use it in intravascular monitoring of glucose levels during hemorrhagic shock. Outer coatings of polyurethane, polyvinylchloride and cellulose acetate have been employed to extend the linear range of the sensor. Sensor response has been evaluated in serum and at 37°C.

2. Experimental

2.1. Reagents and materials

Glucose Oxidase (GOD, E.C.1.1.3.4, activity 250 EU mg^{-1}, from *Aspergillus Niger*) was from Sigma

(St. Louis, MO), and used without further purification. The 1,3-phenylenediamine (also known as 1,3-diaminobenzene) monomer and platinum wires of 0.25 mm diameters were obtained from Aldrich (Milwaukee, WI). Stainless steel 18 gauge needles (outer diameter 1.27 mm) manufactured by Becton Dickinson (Rutherford, NJ) were used. The Nafion® perfluorinated ionomer was obtained from Aldrich as 5 wt% solution and used after dilution to 0.5% using equal amounts of 2-propanol and distilled water. D-Glucose, anhydrous (Baker Analyzed, Phillipsburg, NJ) was used as a stock solution (20 g/l) in phosphate buffer, prepared at least one day before measurements for mutarotation. The interferents used: 4-acetamidophenol (acetaminophen; APAP; *N*-acetyl-*p*-aminophenol), uric acid (2,6,8-trioxypurine), L-ascorbic acid, and glycine were from Sigma. Urea was obtained from Fisher (Fair Lawn, NJ). The polymer coatings were: cellulose acetate from Eastman Kodak (Rochester, NY), polyurethane SG80A from Thermedics (Woburn, MA) and polyvinylchloride 140 × 31 from BF Goodrich (Avon Lake, OH). All other reagents used were of analytical grade.

2.2. *Sensor preparation*

A stainless steel needle (18 gauge) was cut at both ends to remove the plastic cap and the pointed end, and the ends were smoothed using files and fine sand paper. The needle was cleaned in concentrated nitric acid, washed with distilled water and dried. A platinum wire was cleaned in concentrated nitric acid and treated in a propane flame to form a smooth shape or a bulb at one of the ends. The schematic of the prepared sensor is shown in Fig. 1. The platinum wire (1), except the bulb end, was insulated by dipping in silastic (2). The insulated platinum wire was then inserted into the body of the needle (3) and affixed with cyanoacrylate glue. Before the electropolymerization process, the platinum wire was electrochemically treated by cycling between −1.0 and +2.0 V versus a silver/silver chloride reference electrode for 1 h.

The electropolymerization of 1,3-phenylenediamine and the incorporation of the enzyme particles was carried in a solution containing 3–5 mg of 1,3-phenylenediamine, 20 mg of glucose oxidase and 20 mg of enzyme immobilized on ULTI carbon powder in 9 ml of phosphate buffer solution (pH 7.4) together with 1 ml of Nafion solution. This preparation process was performed potentiostatically at +0.65 V versus a silver/silver chloride reference electrode for 15 min. Another layer of poly(1,3-phenylenediamine) was grown over the sensor from a solution containing 5 mg of 1,3-phenylenediamine for 10 min. The sensor was washed thoroughly in a stirred buffer solution overnight.

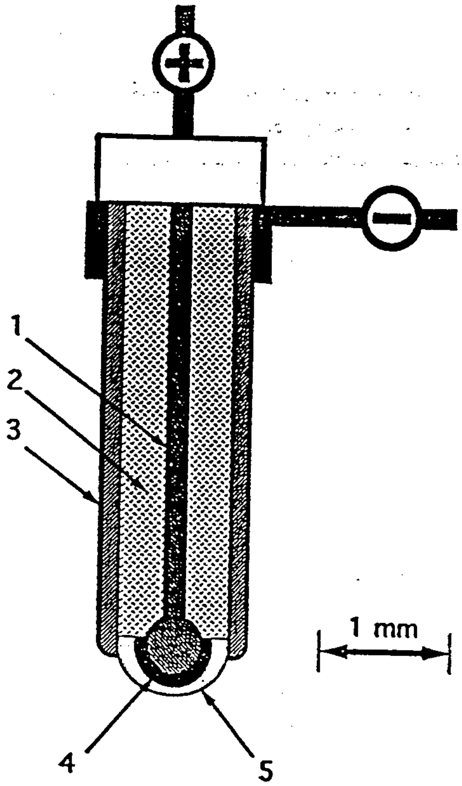

Fig. 1. Schematic cross-section of the needle glucose biosensor: (1) platinum wire working electrode (anode); (2) insulation of the working electrode; (3) stainless steel needle counter electrode (cathode) and sensor body; (4) enzyme incorporated in poly(1,3-phenylenediamine) film; (5) external polymer coating.

A variety of different polymer coatings were employed in order to attempt to extend the linear range of the prepared sensors. Different concentrations of polyurethane, polyvinylchloride (PVC), and cellulose acetate coating solutions were investigated. The polymer coatings were obtained by dipping the face of the sensor in the polymer solution for 20 s. The sensor was then held vertically and dried in air. In the case of the cellulose acetate coatings the sensors were dried for 3 h, while polyurethane and PVC coated sensors were dried for 1 h. The sensor was then left to soak overnight in a phosphate buffer solution.

2.3. *Apparatus and procedure*

The needle sensors were tested in vitro in a magnetically stirred, thermostated measuring cell (volume 50 ml). A two electrode circuit was used, the platinum wire as a working electrode polarized as an anode, and the stainless steel needle body as both reference and counter electrodes, polarized as a cathode. The potential was maintained by a potentiostat (BAS CV-1B, Bioanalytical Systems, West Lafayette, IN). The amperometric signals were recorded on Omnigraphic 100 recorders (Houston Instruments, Austin, TX). The supporting electrolyte was 0.1 M phosphate buffer solution, pH 7.4 containing 0.1 M KCl. Between measurements the sensors were stored soaked in phosphate buffer (pH 7.4) in closed vessels at 4°C. Before measurements the sensors were polarized for at least 10 min at the working potential to establish the background current.

Hydrodynamic voltammograms were obtained by manually varying the potential of the platinum electrode versus the stainless steel electrode from +0.3 V to +0.9 V in 0.05 V steps and recording the steady-state value of the anodic current. During the experiments the electrolyte solution in the measuring cell (volume 25 ml) was continuously stirred by a magnetic stirrer.

Calibration curves –the dependence of the steady state anodic current on glucose concentration –were obtained when the glucose concentration was varied by consecutive additions of glucose from stock solution to the measuring cell in 2.2 mM concentration increments. The calibration curves were obtained with the sensor operating being thermostated at 25 or 37°C.

The sensor response to interfering substances was tested by additions of five common physiological interferents. Stock solutions of 10 mM glycine, 5 mM urea, 6 mM ascorbic acid, 20 mM acetaminophen and 15 mM uric acid were used. Aliquot amounts of these solutions were injected into the measuring cell to give the normal physiological levels in human plasma of these substances, which are: 0.125 mM for ascorbic acid, 0.33 mM for uric acid, 0.5 mM for urea, 0.5 mM for glycine and 0.13 mM for acetaminophen [30].

Calibration curves in blood plasma were obtained using plasma samples prepared from heparinized fresh bovine blood by centrifugal separation. Glucose levels in these samples are measured by routine clinical methods (Beckmann Glucose Analyzer) and then varied by addition of small volumes of glucose from stock solution to the measuring cell to avoid plasma dilution.

Reproducibility of the sensor response was tested by recording the current signal to alternatingly varying glucose concentrations between 2.8 mM (50 mg/dl) to 5.6 mM (100 mg/dl), and between 5.6 mM (100 mg/dl) to 11.1 mM (200 mg/dl).

The glucose concentration profile during hemorrhagic shock [31] was simulated in vitro by varying the concentration of glucose in phosphate buffer solution by step changes from 5.6 mM (100 mg/dl) to 16.7 mM (300 mg/dl) and then, after 20 min from 16.7 mM (300 mg/dl) to 1.7 mM (30 mg/dl). During these experiments the transient amperometric response of the sensor were continuously recorded.

3. Results and discussion

Steady-state (hydrodynamic) voltammograms were obtained in order to estimate a working potential for the prepared needle sensors. Fig. 2a shows typical hydrodynamic voltammograms of a needle sensor in the presence (4.4 mM, curve 1) and the absence (curve 2) of glucose in the cell. In glucose solutions a significant increase of the anodic current at potentials more positive than +0.35 V occurs. This anodic current did not reach an observable plateau due to the increase of the background current at higher potentials. The difference between the anodic current in glucose solutions and the background current (sensor response) is plotted against the applied potential (shown in Fig. 2b). It is clear that a plateau is reached at potentials more anodic than +0.7 V, forming the range of diffusion limited

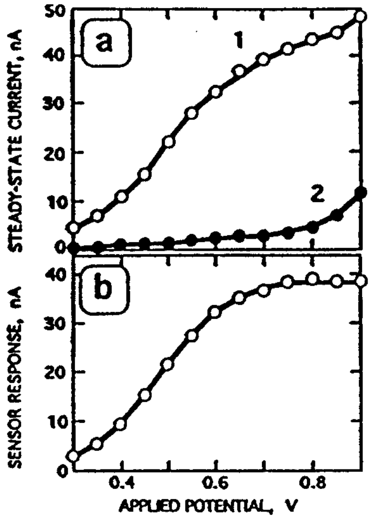

Fig. 2. Hydrodynamic voltammograms of the needle-type biosensor (platinum electrode vs. stainless steel needle): (a) in 4.4 mM glucose solution (curve 1) and the background curve in blank phosphate buffer (curve 2); (b) resulting dependence of the sensor response vs. applied potential (subtraction of curve 2 from curve 1).

electrooxidation of hydrogen peroxide. A working potential of +0.7 V was chosen, and all sensors were operated polarized at this potential.

3.1. Sensors response to glucose

The method of calibration curves was chosen to evaluate sensor performance. Calibration curves were obtained for both uncoated and polymer coated sensors. Analysis of the calibration curves of the sensors having no external polymer layer show a non-linear dependence of the response to glucose concentration over the whole range from 2.2 mM to 40 mM. Linearization of this response was obtained by plotting the data in semi-reciproach coordinates. For example, the non-coated sensors show a typical linear dependence in the Eadie-Hofstee coordinates (current vs. current to concentration ratio). From the linearization of the calibration curves in semi-reciproach coordinates, it can be concluded that Michaelis-Menten kinetics of the enzymatic reaction is the rate-limiting factor in this case. This shows that the poly(1,3-phenylenediamine) matrix itself does not provide diffusion limitations to the glucose flux. The immobilized enzyme in the prepared needle sensors show an average $K_{\mathrm{M\ app}}$ of 9.325 mM ±1.283 mM (standard deviation).

One aspect of using the 1,3-phenylenediamine as an entrapping matrix is the difficulty of obtaining a linear response in the hyperglycemic range (above

Table 1

Polymer coatings and their effect on the linearity, sensitivity and response time of the prepared sensors

Coating	Concentration (mg %)	Linearity (mM)	Sensitivity (nA/mM)	Response time (s)
Cellulose acetate	0.2	2.2	10.8	69
	1	2.2	5.4	69
	5	11.1	3.2	120
	7.5	17.7	2.3	120
	10	26.6	1.6	183
	15	–[a]		
Polyurethane	0.5	2.2	34.7	19
	1	2.2	18.9	19
	5	2.2	13.9	22
	7	13.3	4.1	22
	10	17.7	2.2	24
	12	31.1	1.4	24
	15	–		
Polyvinylchloride	0.2	2.2	23.8	32
	1	2.2	17.6	32
	5	8.8	12.2	34
	8	17.7	2.7	35
	10	37.7	1.8	35
	12	–		

[a] No response.

10 mM). During monitoring of glucose levels in hemorrhagic shock, glucose levels may rise to hyperglycemic levels, up to 20 mM [31]. The application of additional (external) diffusion limiting membranes as polymer coatings is shown to be a way to obtain a linear dependence of the sensor signal versus analyte concentration.

Table 1 shows a summary of the sensors characteristics obtained with sensors employing three different polymer coatings, namely; cellulose acetate (CA), polyurethane (PU) and polyvinylchloride (PVC). The table presents a variety of concentrations of the polymer coating solutions and the resulting parameters of the calibration curves of the sensors: linearity (as the upper limit of the calibration curve linear range), sensitivity (slope of the linear portion of the calibration curve) and response time. It should be noted that any coated sensors showing a non-linear response were assumed to have a linear range of 2.2 mM as the linearity below this range was not investigated. It can be seen that extended linearity is not obtained with any of the three polymers below a polymer coating concentration of 5%. At a concentration of 5% of PVC and CA solutions and 7% of PU solutions a linearity of up to 8.9, 11.1 and 13.3 mM respectively was obtained. Increasing the polymer coating solution concentrations extends the linear range of the sensor response further, but is accompanied by a corresponding decrease in the sensor sensitivity. This can be explained by assuming that increasing the polymer coating concentration produces a thicker or less porous coating. Increasing the coating thickness or decreasing its porosity limits the flux of glucose and thus decreases the amount of hydrogen peroxide produced, reducing the response to a given glucose concentration.

The diffusion-limited operation of the sensor response is also confirmed in the calibration tests performed with different agitations in the measuring cell –there is no difference in the sensor response when the stirring speed is varied from 100 to about 600 rpm.

Fig. 3 presents an example set of the calibration curves, obtained with sensors coated with a PVC external layer. This figure illustrates the transition from the kinetically controlled calibration curve of the uncoated sensor (curve 1) to the diffusion controlled calibration dependence for the sensor coated with PVC (curves 2, 3 and 4). It can be seen that the uncoated sensor has the highest sensitivity of 30.6 nA/mM, and no linear range of response to glucose. Increasing the concentration of the PVC solution to 10% yields a linear range of 37.8 mM and decreases the sensitivity to 1.8 nA/mM. Increasing the PVC coating solution concentration also results in a corresponding decrease in the upper limit of the current response of the sensor to high glucose concentrations.

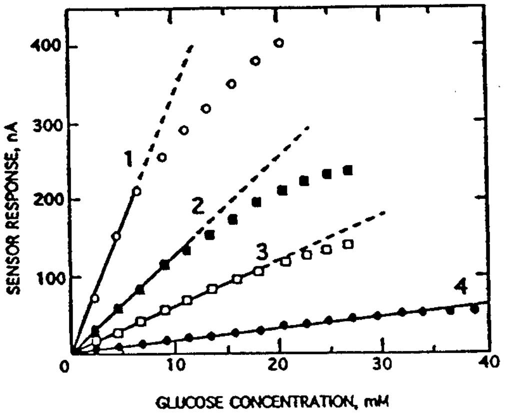

Fig. 3. Calibration curves of the needle glucose sensors with PVC external polymer coating formed from polymer solutions with different concentrations. (1) Non-coated sensor calibration curve; (2) calibration curve of a sensor coated from a 5% PVC solution; (3) calibration curve of a sensor coated from a 7% PVC solution; (4) calibration curve of a sensor coated from a 10% PVC solution.

The response time (defined as time to reach 95% of the steady-state value of the current signal) is also affected by the use of the coatings. Table 1 presents typical response times obtained for sensors with different polymer coatings during calibration tests. It was noted that the response time of each coating does not depend on the concentration when the sensor operates within its linear range. The response time in this case is a function of the concentration step-change only. The response time (for the concentration step-change of 2.2 mM) for the sensor with PVC coating is 34 s; for the PU coated sensor it is 23 s and for the CA coated sensor 3 min. It can be seen from the Table that increasing the concentration of PU or PVC coating solutions has a negligible effect on the sensor response time. While in the case of CA, increasing the CA coating solution concentration results in a corresponding increase in the sensor response time. At the highest glucose concentrations, when kinetics become the rate-limiting factor, the response time increases with increasing concentration of glucose.

Since the ultimate use of the sensor will be in intravenous implantation, the sensor operation and response were evaluated in vitro at 37°C. Fig. 4 shows calibration curves of the sensor operation at 25 and at 37°C, for the sensors coated with PVC (Fig. 4a), CA (Fig. 4b) and PU (Fig. 4c). It can be seen from this figure that sensor operation at 37°C results in a decrease in the linear range of the calibration curves of the sensors coated with the three different polymer coatings. In Fig. 4a, the linearity of the PVC coated sensors decreases from 37.7 mM at 25°C (curve 1) to 22.2 mM at 37°C (curve 2). Fig. 4b shows that the linearity of the CA coated sensors decreases from 28.9 mM (curve 1) to 15.6 mM (curve 2), and the PU coated sensors show a decrease in linearity from 31.1 mM (curve 1) to 20 mM (curve 2). This could be explained the increased enzyme activity at the elevated temperature as well as the widening of the polymer coating pores (thus increasing the glucose flux).

The reproducibility of the sensor response was tested by varying the glucose concentration in the measuring cell alternately between 2.8 and 5.6 mM with concentration step-changes of 2.8 mM, and between 5.6 and 11.1 mM with step changes of 5.6 mM. The sensors monitored the glucose level in the cell for two hours in between the concentration changes. Continuous testing for more than five days was performed, the sensors being left overnight operating at lowest glucose concentrations. It was found that for every level of glucose concentration, the sensor response attained the same amperometric signal value within an acceptable ±5% error. PVC coated sensors showed a response time of 56 s for a concentration step-change of 5.6 mM, and 38 s for a concentration step change of 2.6 mM. There is no observable difference between the response times for increasing and decreasing concentrations.

The glucose concentration profile during hemorrhagic shock was simulated by a concentration step change from 5.6 mM (approximating the normal physiological value) to 16.7 mM, and after 30 min (the compensatory phase) an instant decrease from 16.7 mM to 1.7 mM (the decompensatory phase).

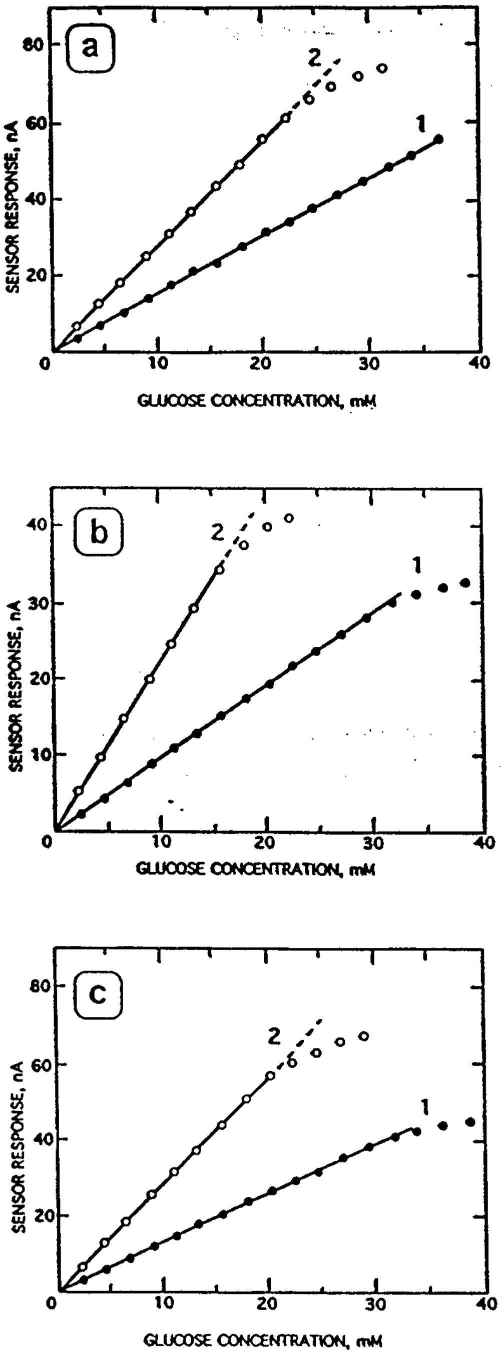

Fig. 4. Calibration curves at 25°C (curves 1) and at 37°C (curves 2) of sensors coated with different polymer layers: (a) sensor coated from a 10% PVC solution; (b) sensor coated from a 10% CA solution; (c) sensor coated from a 12% PU solution.

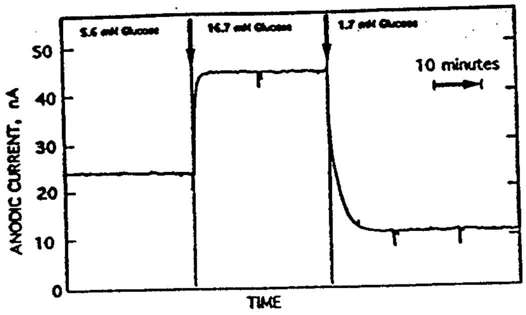

Fig. 5. Protocol of the amperometric current response of the needle glucose sensor to glucose concentration step changes in the measuring cell simulating glycaemia profile during hemorrhagic shock.

Typical transients of the sensor current response to this test are shown in Fig. 5. The PVC coated sensors reach a steady-state value in 1.73 min during the increasing step, and in 5.1 min during the decreasing step of the test. These tests were performed six times during continuous operation of the sensors for one week. The values of the steady-state amperometric response of this sensors were within 10% of values predicted from the calibration curves.

It should be noted that the concentration change during this tests is a step change, as opposed to the gradual concentration changes in the physiological processes. These results show that the sensors are able to respond to the varying glucose concentrations in an acceptable time and with reproducible current values. It can be seen from the data that the use of PVC coatings has an advantage in increasing the linear range while preserving the response time.

3.2. *Interference and blood plasma tests*

Fig. 6a shows protocols of the sensor response to the consecutive addition of urea, glycine, acetaminophen, ascorbic acid and uric acid to the measuring cell in concentrations corresponding to their physiological levels. The response to the interfering substances in the presence and absence of glucose was studied, and curve 1 shows a protocol obtained when the sensor monitors 6.6 mM glucose. After the addition of the interferents, glucose concentration is further increased in 2.2 mM concentration increments. Curve 2 of Fig. 6a presents a protocol of the sensor recording when interferents are added to the measuring cell followed by further additions of glucose to obtain a calibration curve in interference solution. Data obtained in these two experiments are compared with the sensor calibration curve in buffer in Fig. 6b. Fig. 6b is a comparison between a calibration curve obtained in blank phosphate buffer (curve 1) and two calibration curves obtained in the

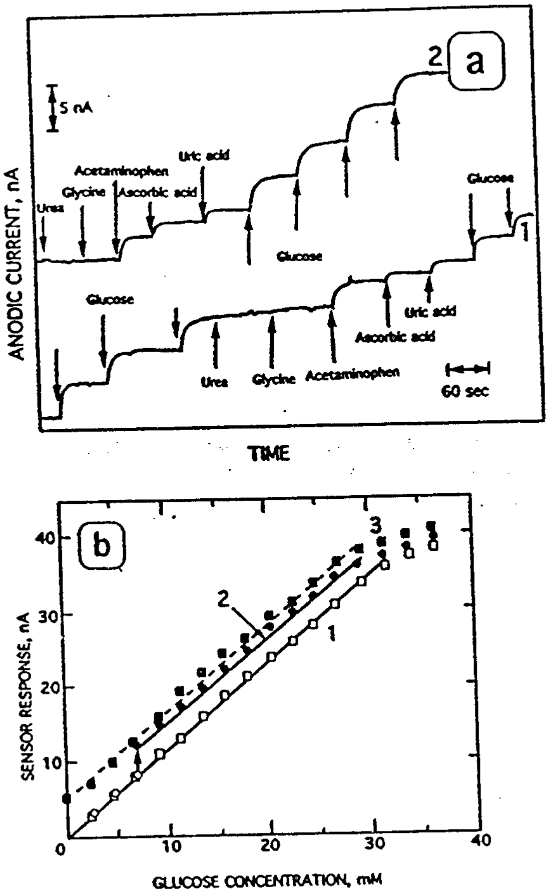

Fig. 6. PVC coated sensor response to common physiological interferents. (a) Protocol of the sensor response to addition of urea, glycine, acetaminophen, ascorbic acid and uric acid to the measuring cell containing glucose (curve 1). Protocol of the sensor response to additions of the same interferents to the measuring cell with further addition of glucose (curve 2). (b) Calibration curves of the sensor from (a) in absence of interferents (curve 1); calibration of the sensor obtained in absence of the interferents up to 6.6 mM followed by addition of urea, glycine, acetaminophen, ascorbic acid and uric acid (according to (a), curve 1) and followed by further additions of glucose (curve 2); calibration of the sensor obtained in the presence of above interferents (curve 3).

presence of interferents (curves 2 and 3). One calibration curve (curve 2) obtained by addition of five common physiological interferents (urea, glycine, acetaminophen, ascorbic acid and uric acid), one after the other, followed by further additions of glucose. The second interference calibration curve (curve 3) is obtained by adding glucose in 2.2 mM increments up to 6.6 mM and then addition of the interferents (listed above) followed by the consecutive addition of glucose. It can be seen that the total effect of the five interferents on the current signal is equivalent to addition of a 2.7 mM of glucose. No sensor response to either glycine or urea is noted (Fig. 6a). The sensor response to ascorbic and uric acid is 30% of the sensor response to a 2.2 mM addition of glucose. Another point to be noted is that addition of interferents did not change the slope of the calibration curves, thus indicating that the interferents did not affect the sensitivity of the sensor. The diminished response to interferents can be attributed to the permselectivity characteristics of the poly(1,3-phenylenediamine) film [19] and to the Nafion negatively charged ionomer used in the sensor preparation.

The calibration curve in bovine serum is obtained by a procedure similar to that used when phosphate buffer solution. Fig. 7 presents a comparison between the calibration curve in bovine plasma and two calibration curves in phosphate buffer solution (obtained before and after the plasma test) for a PVC coated sensor. The calibration curve in bovine plasma starts from concentration of glucose 4.7 mM (the actual concentration of the plasma) to avoid dilution. The calibration curves in buffer solution are presented without subtraction of the background current so as to be comparable with the curve obtained in plasma. It can be seen from Fig. 7 that the sensor response in plasma has a smaller slope (hence lower sensitivity) than that obtained in phosphate buffer, yet it is within an acceptable 6% difference. The decrease of sensor sensitivity is probably due to the adsorption of proteins present in the plasma on the surface of the sensor providing additional diffusional limitations.

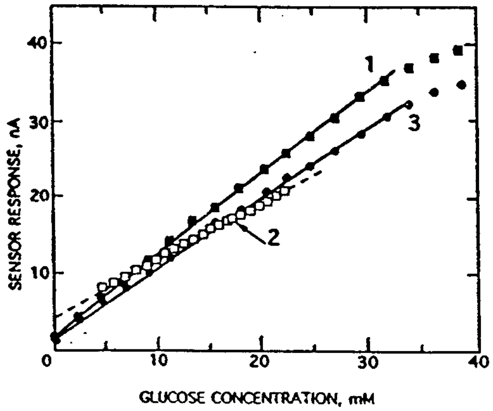

Fig. 7. Calibration curve of glucose biosensor with a PVC coating in phosphate buffer before plasma test (curve 1), calibration curve of the sensor in bovine blood plasma (curve 2) and in phosphate buffer obtained after plasma test (curve 3).

3.3. Sensor life times

The stability of the sensors over time is recognized as one of the most important factors with respect of their practical application. Two aspects of the sensor's life-times have been studied: the storage life-time (shelf life), and the long-term operational stability.

In order to estimate the storage life-time, three sets of sensors six of each of PVC membrane, PU and CA coated. These sensors were then stored in closed vessels (soaked in phosphate buffer) at 4°C and calibration curves after one week of storage of time (daily, 5 times a week). It should be mentioned that the presented data are from the first measurement for each of the sensors in the set. Storage time is counted from the day of sensor preparation. It was observed that in each of the sets, sensors demonstrated typical values of their sensitivity and linear range. Within each series the difference in the parameters of the calibration curves do not exceed a variation of ±10%. These sets of sensors were tested during a period of 2 weeks (14 days) and no practical change of their performance (within ±10%) has been seen.

The long term operational stability of the sensors was evaluated along with the reproducibility tests. Continuous testing for more than seven days were performed while sensors were left operating overnight at the lowest glucose concentrations. It was found that for the seven day period the sensor response maintains the same amperometric signal value within an acceptable ±5% error limit.

4. Conclusions and future work

This communication presents initial results on the development of a needle glucose biosensor for monitoring of glucose levels during the hemorrhagic shock in trauma patients. The sensors employ commercially available cellulose acetate, polyurethane or polyvinylchloride as a diffusion membrane for glucose. Sensor performance in model phosphate buffer solutions and in bovine plasma was investigated, and sensors were characterized by their response time, sensitivity and linear range of the calibration curves. Long term stability studies of the sensors show that they did not change their main characteristics when stored for at least two weeks, or when operated continuously for 7 days. Additional experiments on the long-term stability of the sensors and their shelf life time are still in progress. Further work will include evaluation of sensor response in whole blood in vitro. Glucose level monitoring during hemorrhage shock will be performed with these sensors when implanted intravenously in laboratory animals.

Acknowledgements

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APPENDIX 4

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Needle-Type Sensor for Whole Blood Glucose Monitoring

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ABSTRACT

A new surface process technology employing electrochemical fixation of a bioactive substance (enzyme and heparin) to a sensor electrode was developed to provide biocompatibility and functionality. The fabrication process includes electro-entrapment of glucose oxidase and heparin on a platinum electrode by using 1,3-phenylenediamine co-deposition. PVC was used as the outer coating in order to extend the linear range. The sensors show a sensitivity of 3 nA/mM and a linear range from 40 to 400 mg/dL at 37 °C. when tested in whole blood. The sensors are characterized by a fast response. Sensors show a minimum change in their performance when stored inactive in buffer for 12 weeks. When tested at physiological glucose levels, the sensors demonstrate satisfactory low interference from common interfering substances. This technology seems very promising for the preparation of future implantable intravascular biosensor.

Needle-Type Sensor for Whole Blood Glucose Monitoring

INTRODUCTION

Glucose biosensors have received much attention because of their many applications in clinical chemistry, biotechnology and the food industry. Research efforts have been in particular directed towards the development of an amperometric sensor for glucose, due to the importance of glucose monitoring for diabetic patients [1]. Success in this research has reached the level of short term human implantation [2].

An important step in the fabrication of glucose biosensors is the immobilization of glucose oxidase (GOD) enzyme. Various methods have been used for this process: cross-linking with glutaraldehyde, chemical immobilization on carbon carriers [3], entrapment in polymer layers [4-6], in metal matrixes [7-8] or electrochemically grown polymer films on the electrode surface [9].

Entrapment in electrochemically polymerized film is a simple and attractive approach for the immobilization of enzymes on electrode surfaces. The process, which is also referred to as electrochemical immobilization, involves the electrochemical oxidation of a suitable monomer from a solution containing enzyme to form a conducting or non-conducting polymer on the surface of the electrode. The solution for electropolymerization should preferably be an aqueous solution with a neutral pH to facilitate the incorporation of the biological component into the polymer film in an intact form.

The process of electropolymerization can be controlled by the electrode potential, and allows accurate control of the polymer film thickness and hence the amount of enzyme entrapped in close proximity to the electrode surface. In addition, since electropolymerization occurs locally at the electrode surface, it can be used to confine an enzyme precisely at the polarized electrode without cross-immobilizing it on a neighboring electrode. This property makes it a suitable procedure for the fabrication of arrays of enzyme microelectrodes.

It is also possible to use this technique as a method for the preparation of multilayer structures, either with one or more enzymes layered within a single polymer, or with one enzyme within a multilayered polymers.

As a consequence of these useful characteristics, the entrapment of enzymes within electropolymerized films has become an increasingly important technique. Consequently, this technique has found a wide range of applications in the fields of both biosensors and molecular electronics, as well as in the study of heterogeneous enzymic catalysis within membranes.

Both conductive polymers (like polypyrrole) and non conductive polymers (like phenylenediamine) have been used to entrap the enzyme on the electrode. Use of nonconducting polymers is advantageous because the polymer growth is self limiting, producing thin polymer films. This is of particular importance in the design of diffusion limited sensors with short sensor response times. Thin films also provide a higher enzyme content in close proximity to the electrode surface, leading to a larger sensor response and a high sensitivity. It has been reported that non-conducting polymers also have a characteristic permselectivity that greatly decreases the effect of common physiological interferents on the sensor response [10-11,13]. Yacynych et al. [12] demonstrated that the oxidation of non-conducting polymer 1,2-phenylenediamine was irreversible, resulting in the formation of an insulating polymer film completely covering the electrode surface and protecting the transducer against fouling species. Poly (1,3-phenylenediamine) has successfully been employed in the construction of laboratory prototype of glucose sensors [14-16].

Heparin is a natural sulfated glycosaminoglycan consisting largely of alternating O- or N-acid (D-glucuronic or L-iduronic) and D-glycocyamine residues. Heparin exerts its anticoagulant activity by accelerating (in some cases 1000-fold) the inactivation by antithrombin III of the activated serine proteases involved in the coagulation cascade; Factors XIIa, XIa, IXa, Xa, and most importantly thrombin. with thrombin inactivated or its formation prevented. Fibrinogen is not converted to fibrin and the fibrin gel or “clot “ does not form. The antithrombotic activity refers to the *in vivo* ability of heparin to inhibit thrombus formation in experimental or clinical situation exposing to the risk of developing thrombosis.

The response of the intravascular electrode would be drastically changed due to the formation of thrombi on foreign surfaces. Immobilization of heparin on artificial surfaces has been extensively explored as a possible method for creating a thromboresistant surface. For long term use of such devices, heparin should be immobilized by a stable bonding procedure. However, the attempts to bind heparin covalently, that have been made so far have had little success, presumably because the chemical and structural characteristics responsible for the anticoagulant activity of heparin have not been considered when choosing the methods for binding. The lack of methods for evaluation of the ultimate blood contact properties is another factor which has hampered progress.

Heparin-bonded polymer surfaces prevent platelet adhesion and take up thrombin, which is then inactivated in the presence of plasma. These are important characteristics for thromboresistance. Reasonable amounts of heparin on the surface result in a 1,000-fold reduction in thrombin concentration in close proximity to the treated surface [17]. Nakayama et al.[18] demonstrated the immobilization of heparin on poly(ethylene-tetrephthalate), and the immobilization of glucose oxidase onto the surface of a carbon fiber electrode to be used as glucose sensor. The surface process technology developed may be applied to medical devices. A new non-thrombogenic surface prepared by selective covalent binding of heparin via a modified reducing terminal residue [19] was reported. The heparinized surface releases insignificant amounts of heparin and can be regarded as stable. The blood contact properties as studied in vitro revealed that the surface was highly thromboresistant in terms of reduced platelet adhesion, surface catalyzed adsorption and inhibition of thrombin and capacity to prevent clotting of non-anticoagulated blood.

In the present paper we use electro-deposition method to entrap heparin onto the surface of a glucose enzyme electrode. Our goal is to develop a sensor which could be used to determine the glucose concentration in the whole blood, and is suitable for intravascular monitoring.

EXPERIMENTAL

Reagents and Materials

Glucose Oxidase (GOD, E.C.1.1.3.4, activity 162 EU/mg, from *Aspergillus Niger)* and ß-D-Glucose were from Sigma Chemical Co.(St. Louis, MO), Ultra Low Temperature Isotropic (ULTI) carbon powder was from Carbomedics, 1,3-phenylenediamine (99+ % purity), and Nafion (perfluorinated ion-exchange polymer, 5 wt.% solution) were from Aldrich Chemical Co. (Milwaukee, WI). Platinum wire (0.5 mm and 1.5mm in diam.) and silver wire (0.127 mm diam.) were from Alfa Chemical Co. (Ward Hill, MA). Epoxy-patch was from the Dexter Corporation, (Seabrook, NH), Heparin (1000 units/1 ml), Elkins-sinn, Inc. (Cherry Hill, NJ).

Sensor Preparation

H_2O_2 sensor design and fabrication.

Fig. 1.a presents the schematic diagram of the H_2O_2 electrochemical transducer and the needle-type biosensor fused on it. The working electrode was constructed by sealing the platinum wire in the center of the polyethylene tube with epoxy. Copper wire (the current collector) was soldered to the platinum and extended outside the tube for electrical connection. The epoxy resin was cured in the oven at 60 °C for 2 hours. The working electrode surface was sanded flush and polished. Silver wire (about 10 cm long) was wrapped around the tip part of the electrode and served as reference (counter) electrode. The platinum working electrode was polarized as an anode and the silver counter electrode was polarized as a cathode in a two electrode system.

Enzyme immobilization

The schematic of the sensor's working electrode surface is shown in Fig. 1.b. The enzyme GOD was entrapped in an electrochemically grown poly(1,3-phenylenediamine) film. The electropolymerization of 1,3-phenylenediamine and the incorporation of the enzyme particles was carried out in a solution containing 3-5 mg of 1,3-phenylenediamine, 20 mg of glucose oxidase and 20 mg of ULTI carbon powder in dispersed in an electrolyte consisting of 9 mL 0.1 M sodium phosphate buffer solution (pH 7.4) containing 0.125 M KCl and 1 mL of Nafion solution. This preparation process was performed potentiostatically at +0.7 V versus a silver/silver chloride reference electrode for 15 minutes. An additional coating of poly(1,3-phenylenediamine) was applied over the enzyme containing layer. This coating was formed from a solution of 3-5 mg of 1,3-phenylenediamine. This process was performed potentiostatically at + 0.70 V versus a silver/silver chloride reference electrode for 10 minutes. As a result the carbon-enzyme layer (Fig. 1.a) was formed.

Heparinization of the electrode

The process of electrochemical heparinization was carried out in 20 ml buffer solution (pH 7.4) containing 30 mg of 1,3-phenylenediamine and 2.5 mL heparin potentiostatically at +0.7 v versus a silver/silver chloride reference electrode for 10 minutes instead of the coating layer described above. This procedure forms the heparin layer over the carbon-enzyme layer on the electrode surface (Fig. 1.a).

External polymer coating of the electrode

PVC (2-4% in tetrahydrofurane) was applied to the sensor surface as an external protective coating and an additional diffusion control membrane. The membrane was formed over the sensor tip by dip casting. The sensor end was dipped in the polymer solution for 1 second and was then held vertically and dried in air for one hour. By this procedure the external protection/diffusion membrane vas formed (Fig. 1.a). After fabrication the sensors were left in the buffer solution overnight.

Measurement procedure and instrumentation

A potentiostat (BAS CV - 1B Bioanalytical System, West Lafayette, IN) was used to maintain the potential of + 0.7 V between the working and the reference electrode. The amperometric signals were measured and recorded on a X-T recorder (Omnigraphic 100 Houston Instruments, Austin, TX).

The sensor response to glucose was measured in a glass cell (volume 50 cm^3) thermostated at 25 °C or at 37 °C and stirred by a magnetic stirrer. Phosphate buffer solution (pH=7.4) containing 0.125 M KCl was used in the tests and the glucose concentration was varied by consecutive additions of glucose from a stock solution (20 g/L) .

The sensor response to glucose in the whole blood was obtained in a 25 mL glass cell containing 20 ml whole Bovine blood. Bovine blood sample was freshly obtained from a local slaughterhouse and the initial blood glucose level was measured by a standard clinical device (Hemo Cue, Stockholm, Sweden). After thermostating at 37 °C, glucose blood level was varied by consecutive additions of glucose from a stock solution of 20 g/L. During the experiment the blood sample was stirred by a magnetic stirrer.

RESULTS AND DISCUSSION

Cyclic voltammograms were obtained in order to estimate a working potential for the prepared electrodes. The cyclic voltammograms recorded at several glucose concentrations showed an increase of the current in the potential range of hydrogen peroxide oxidation. In glucose solutions, a significant increase of the anodic current at potentials more positive than + 0.35 V occurred. In theses cases the anodic current reached an observable plateau at potentials more positive than + 0.55 V. The saturated current value in the plateau range increased with the increase of the glucose concentration. From the cyclic voltammetry studies a working potential of +0.7 V was chosen, and all sensors were operated polarized at this potential during the constant potential amperometry.

The Selection of 1,3-phenylenediamine Concentration

Fig. 2 presents two calibration curves (dependencies of the steady-state amperometric response of the sensor to consecutive increase in glucose concentration in the measuring cell) for one representative sensor obtained before and after the procedure of heparinization. This Figure demonstrates the performance of the non-coated sensor, having only a carbon-enzyme layer on the working electrode surface. Fig. 2 shows the effect of the heparinization on the sensor response. From this Figure it can be seen that after the heparinization the sensor response apparently decreased in comparison with the response before this procedure. Such a test (comparing the calibration curves before and after the heparinization) was used as a criteria as to whether enough heparin was deposited on the electrode surface. It was found that in the cases with the greatest decrease in the signal after heparinization, the sensor, when tested in the whole blood, would not produce any current signal from the addition of the glucose.

The concentration of the monomer - 1,3-phenylenediamine - in the electropolymerization solution played a key role in the heparinization process. Table 1 presents the effect of the concentration of the 1,3-phenylenediamine on the sensor performance after heparinization. From Table 1 it can be seen that when the concentration of 1,3-phenylenediamine is not high enough, almost no heparin was immobilized on the electrode and when tested in whole blood, the electrode was totally coated by a blood deposit which resulted in a lack of signal. But when the higher concentration of 1,3-phenylenediamine was used, too much 1,3-phenylenediamine and heparin were immobilized on the electrode, which lead to almost no response to the glucose when tested in the whole blood because of overcoating of the sensor surface. So, the optimal concentration range is around 2 mg 1,3-phenylenediamine per mL buffer solution was chosen for further experiments.

Selection of the PVC Coating Solution Concentration

The concentration of the PVC solution from which the external membrane was formed was varied between 0.5% - 4 %. When the concentration was lower, the response was of large magnitude and was characterized by short response time. The linear range, however, was not satisfactory. When the concentration of the coated polymer was higher, the linear range could be extended up to 700 mg/dL, and was accompanied by a significant decrease in sensor sensitivity. According to the performance of the electrode, 2 % PVC solution was selected as the coating polymer concentration.

Performance of the Heparinized Biosensor

An external polymer coating with PVC was used in order to extend the linear range of the sensors calibration curves. Calibration curves were obtained for both non-coated and PVC coated sensors. Fig. 3.a shows examples of calibration curves obtained with a sensor before PVC coating, and a sensor coated with PVC.

Parameters of the sensors calibration curves - linear range and sensitivity - were the focus of this investigation. Analysis of these two parameters of the sensor response is appropriate using the electrochemical Eadie-Hofstee coordinates: sensor response vs. sensitivity. This plot is based on the linearization of Michaelis-Menten equation when reaction rates are substituted with steady-state current fluxes:

$$I_S = I_S^{max} - K_M^{app} (I_S/C_g) \qquad (1)$$

Here I_S is the steady-state current of the electrode (amperometric response), C_g is the concentration of glucose [so that (I_S/C_g) gives the sensitivity], K_M^{app} is the apparent Michaelis-Menten constant of the enzymatic reaction (glucose oxidation by molecular oxygen catalyzed by GOD), and I_S^{max} is the intercept on the current axis corresponding to the maximal rate of the enzymatic reaction. It can be seen from (1) that in the case when the enzymatic reaction is the rate controlling process (kinetic control) the response/sensitivity dependence is linear with negative slope K_M^{app}. This means that the response/concentration dependence (the calibration curve) is hyperbolic (not linear in any concentration range).

Diffusion limitation of the overall processes occurring on the electrode resulted in a strong linearity of the signal/concentration dependencies (calibration curves) following the equation:

$$I_S = nFA\, D^{eff} (C_g - C_o)/L_m \qquad (2)$$

Here n is the number of electrons exchanged, A is the electrode surface area, D^{eff} is the effective diffusion coefficient of the substrate in the membrane, L_m is the membrane thickness, C_g is the bulk substrate concentration and C_o is the surface substrate concentration. When the electrode polarization is sufficiently high and the enzymatic reaction is fast compared to diffusion (diffusion control), then $C_o = 0$ and the amperometric response is directly proportional to the substrate (glucose) concentration. In Eadie-Hofstee coordinates this dependence is shown by a vertical line parallel to the current axis.

Presentation of the experimental data from Fig. 3.a in Eadie-Hofstee coordinates is given in Fig. 3.b. It can be seen that the dependence of the amperometric response on the sensor sensitivity obtained with the sensor before PVC coating is linear with a negative slope according to equation (1). This plot could be interpreted as an evidence of a kinetic control in the entire range of investigated glucose concentrations for the sensor before PVC coating.

The biosensor with an external PVC polymer layer shows a linear dependence of the response to glucose concentration up to at least 400 mg/dL. The polymer coating over the enzyme-carbon layer acted as a glucose diffusion membrane, resulting in the increase of the linear range of the sensor response. It can be seen from Fig. 3.a and Fig. 3.b that the increase in the linear range of the sensors response is associated with a decrease in the sensor sensitivity. The initial sensitivity of the non-coated sensor is 1.5 nA/mg/dL. Sensitivity of the PVC coated sensor is approximately 0.2 nA/mg/dL and is constant in the range from 40 to 400 mg/dL glucose (Fig. 3.b), in agreement with equation (2).

The response time (estimated as the time to reach 95% of the steady - state value of the current signal) is also affected by using the PVC coating. The response time (for a concentration step - change of 40 mg/dL) for the sensor without coating is about 5 s; and for the sensor with a coating is 1.5 min. At the highest glucose concentrations, for the sensor without a coating, the response time increases with increasing concentration of glucose, while for the PVC coated sensors it remains independent of the absolute concentration, being a function on the concentration step-change only. Hence the use of PVC coatings on the electrode with GOD and heparin entrapped in a poly(1,3-phenylenediamine) layer, has the advantages of increasing the linearity of the sensor response over the glucose concentration range of physiological and pathophysiological interest, with an increase of the sensor response time that is still acceptable from a practical point of view.

The effect of temperature elevation was studied. Calibration plots of the sensor with GOD and heparin immobilized by entrapment in a poly(1,3-phenylenediamine) matrix obtained at room and at body temperature are shown in Fig. 4. At 25 °C the linear range of the electrode response is up to 600 mg/dL. At 37 °C the response amplitude increased, but the upper limit of the linear range decreased to about 400 mg/dL. This is probably due to an increase of the external PVC membrane permeability, which may be associated with microcracks or other damage. This result, however, still confirms that the sensor response is linear over the physiological and pathophysiological range of glucose concentration at body temperature.

Sensor life times

The long-term stability of the sensors was evaluated by periodically obtaining calibration curves in buffer solution of the sensors (usually twice a week) from a simultaneously prepared set. From these calibration curves the characteristics of the sensors' response were obtained and the sensor performance was evaluated. Between tests the sensors were stored in the refrigerator at 4 °C. Fig. 5 shows the sensor sensitivity (from the calibration curves) as a function of time of sensor storage since fabrication. Fig. 5 presents data obtained with two individual sensors from the same set (sensors NS1 and NS2). From this Figure it can be seen that the sensitivity remained constant over 11 weeks of storage. A tendency towards increasing sensor sensitivity can be seen. This is associated with a decrease in the linear range But the linear range, however, decreased after 3 weeks of testing. The decrease in the linear range is most evident when the electrodes are tested at 37°C. The increase of the sensor sensitivity with the storage and associated decrease in the linear range is probably due to partial degradation of the PVC coating, during which the effectiveness of the diffusion membrane diminishes.

Interference and the whole blood tests

Fig. 6 shows the sensor response to the consecutive addition of glucose, ascorbic acid (0.1 mM), uric acid(0.4 mM), and acetaminophen (0.2 mM). These concentrations are selected to mimic the maximal concentrations of the substances in human serum [14]. By comparing the response of the interfering substances with that of 6 mM glucose it was found that the electrode has negligible response to ascorbic acid; 4.2% relative response to uric acid and 7.9% relative response to acetaminophen. This shows that poly(1,3-phenylenediamine) can effectively prevent the interference from these substances, and at the same time poly(1,3-phenylenediamine) ensures good sensitivity and repeatability of the sensor response to glucose.

Fig. 7 shows a recording obtained during a whole blood test at 37 °C. The initial blood glucose level is measured to be 85 mg/dL (by a reference method). After every addition, the concentration of bulk glucose was increased by 100 mg/dL. From the Figure it can be seen that sensor responded to the glucose addition. After testing in whole blood, the electrodes were washed and then tested again in buffer solution. It was found that the sensitivity of the sensors remained the same as before the test in the whole blood. This demonstrated that the electrode surface has not been irreversibly clogged by the blood albumin. The sensor signal in whole blood however, has of lower amplitude than that obtained in buffer solution. This could be an indication that there is some adsorption of substances (proteins, lipids) on the electrode surface acting as an additional diffusion barrier for glucose and diminishing the sensor signal.

CONCLUSION

The electrochemical-entrapment immobilization of heparin, useful as a thromboresistant coating, and the immobilization of the glucose oxidase onto a platinum electrode surface, was demonstrated. This technology can greatly improve the sensor response to glucose in the whole blood and may be applied to other devices for intravascular applications.

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FIGURE CAPTIONS

Fig.1 Crossectional schematic of the needle-type glucose biosensor basic electrochemical transducer (a) and a schematic view of the enzyme electrode layered surface stricture (b).

Fig.2 Calibration curves of the needle-type glucose biosensor in phosphate buffer solution (pH 7.4) at room temperature obtained at different stages of the enzyme electrode preparation: before and after the heparinization procedure.

Fig.3 Calibration curves of the needle-type glucose biosensor in phosphate buffer solution (pH 7.4) at room temperature obtained at different stages of the enzyme electrode preparation: before and after coating with a PVC layer (a). Data from (a) presented in electrochemical Eadie-Hofstee coordinates (b).

Fig.4 Calibration curves of the PVC coated needle-type glucose biosensor in phosphate buffer solution (pH 7.4) obtained at room temperature (25 °C) and at body temperature (37°C).

Fig.5 Dependence of the sensor sensitivity (obtained as a slope of a calibration curve) on the time of storage at 4 °C between the measurements for two representative individual sensors (NS1 and NS2).

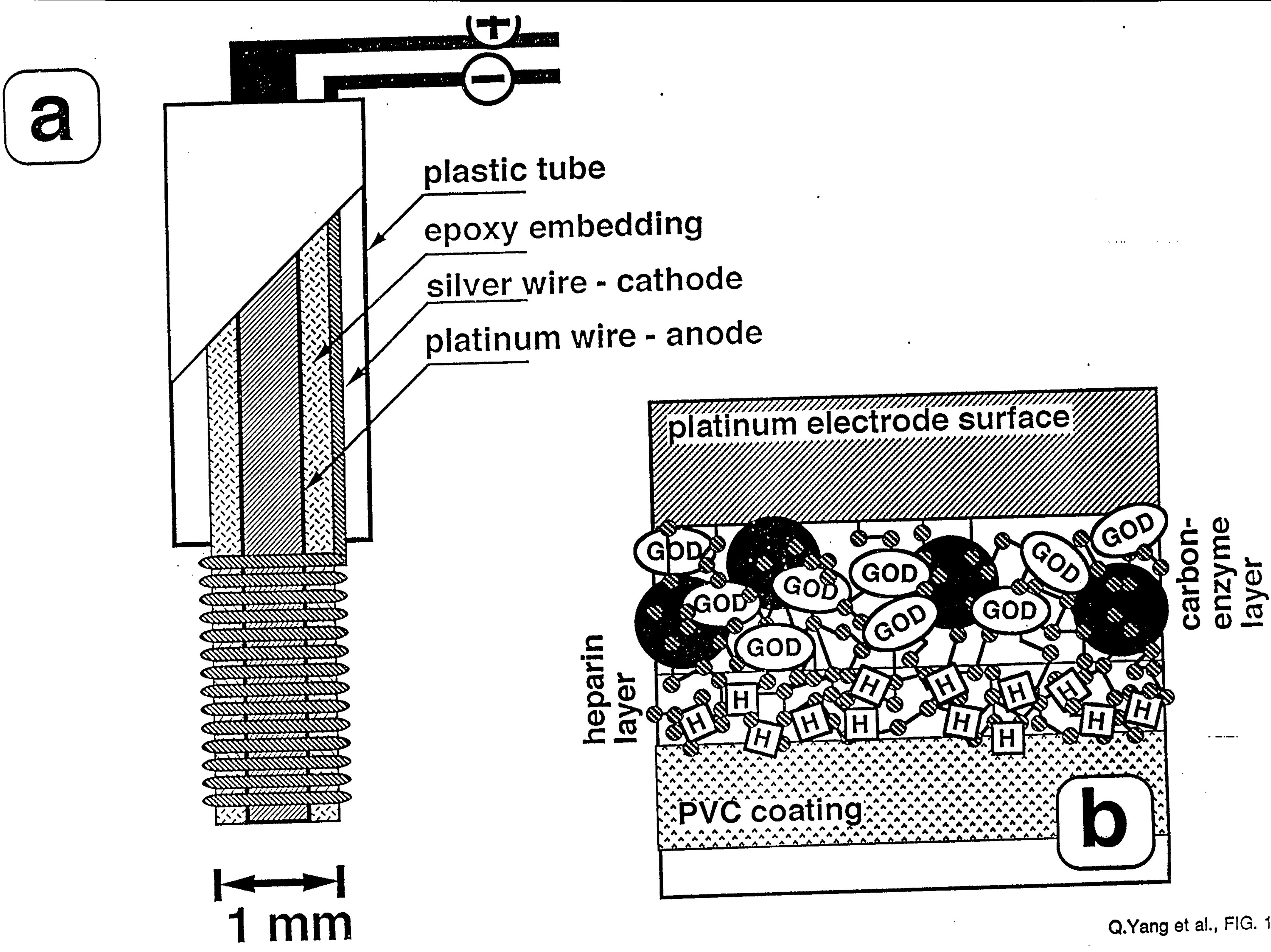
Fig.6 Protocol of the needle-type glucose biosensor response to consecutive additions of glucose in phosphate buffer solution (pH 7.4) with a concentration step change of 20 mg/dL to reach the physiological level of 80 mg/dL glucose followed by consecutive additions of common physiological interferents (ascorbic acid, uric acid and acetaminophen) at their physiological levels.

Fig.7. Needle-type glucose biosensor response to consecutive additions of glucose in whole blood (initial glucose level of 85 mg/dL) with a concentration step change of 100 mg/dL.

Table 1. The effect of the 1,3-phenylenediamine concentration during the heparinization of the electrode on the sensor response to glucose in whole blood

1,3-phenylenediamine concentration [mg/mL]	Sensor response to glucose in blood [nA/200 mg/dL]
0.5	0.0
1.0	5.0
2.0	10.0
5.0	0.0

*****


a
(+)
(−)
plastic tube
epoxy embedding
silver wire - cathode
platinum wire - anode
1 mm
b
platinum electrode surface
GOD
H
carbon-
enzyme
layer
heparin
layer
PVC coating

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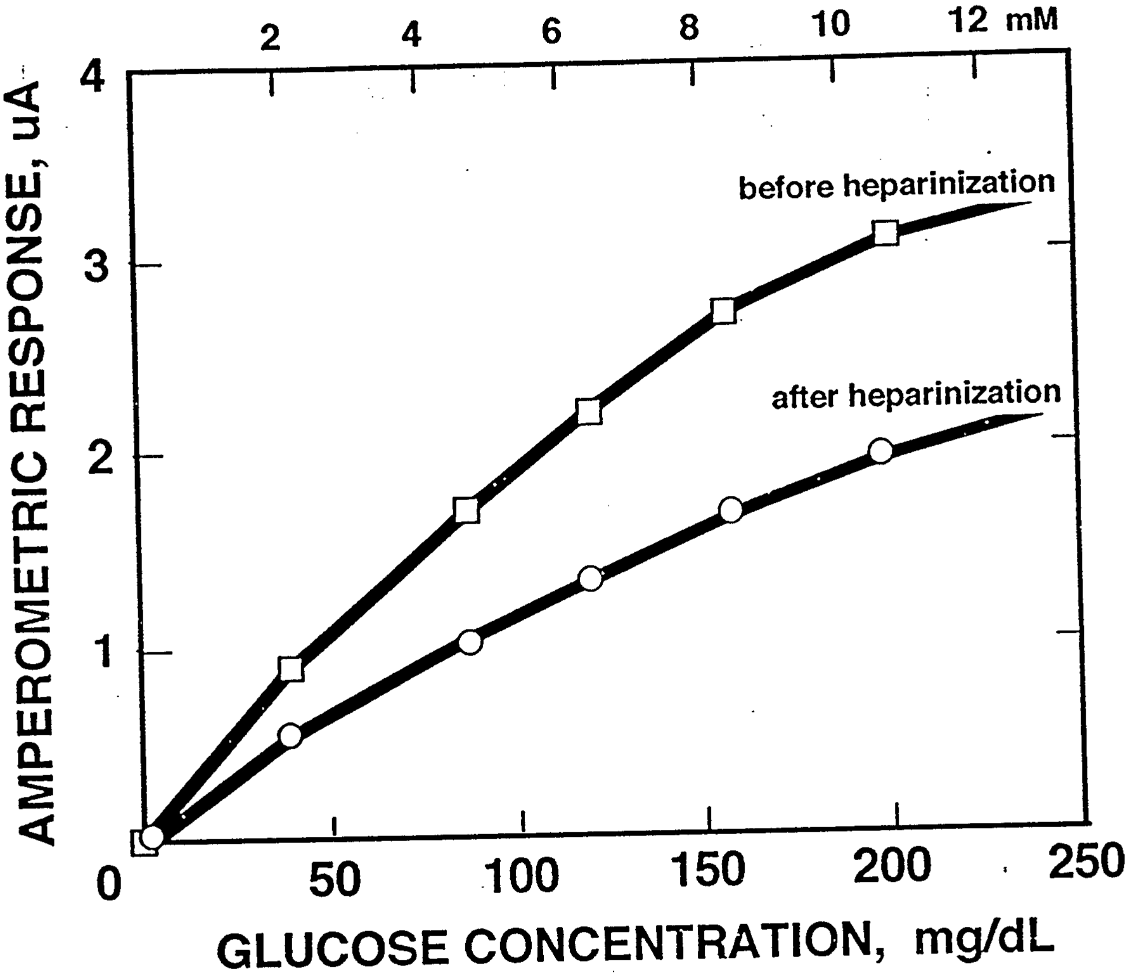
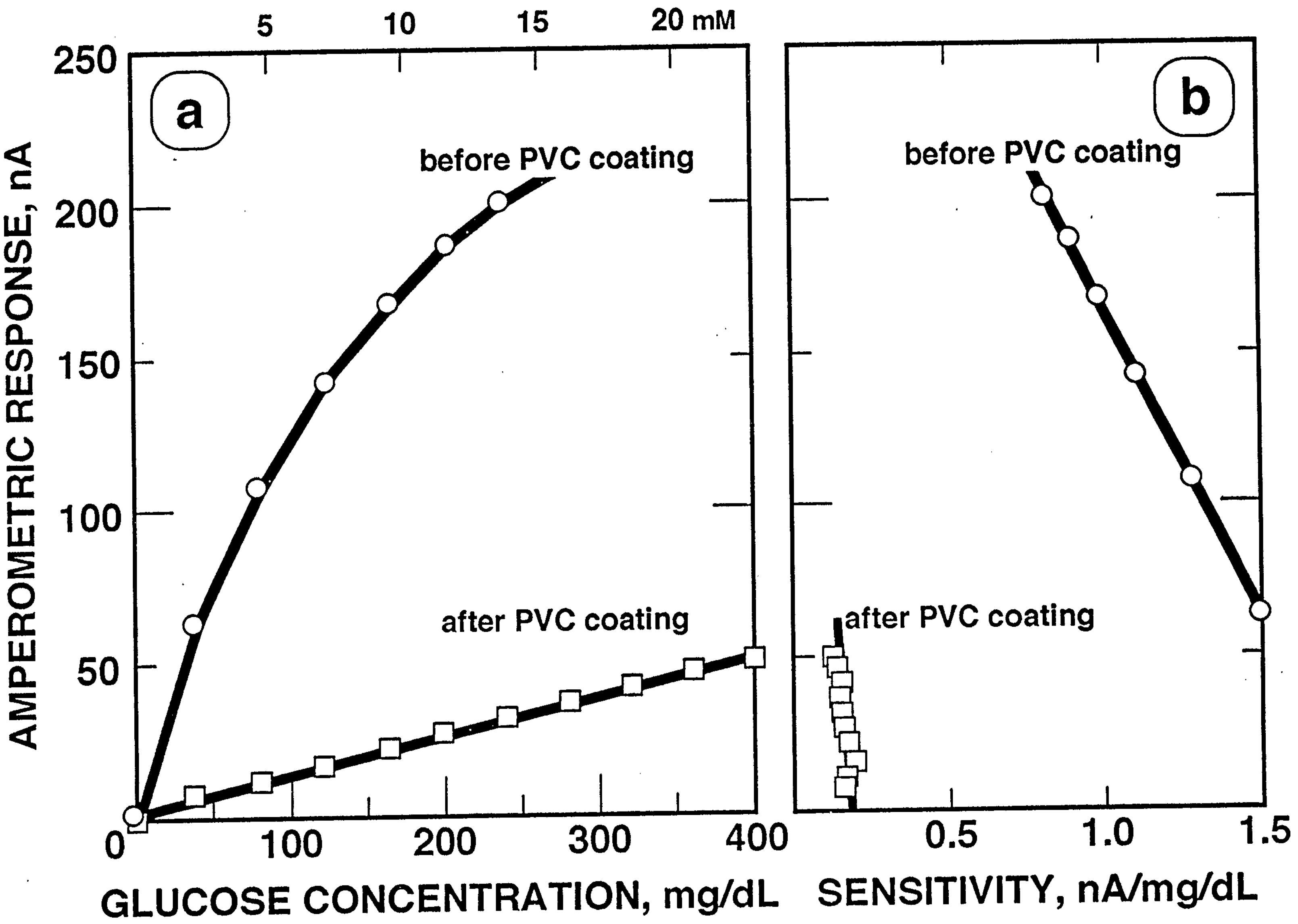
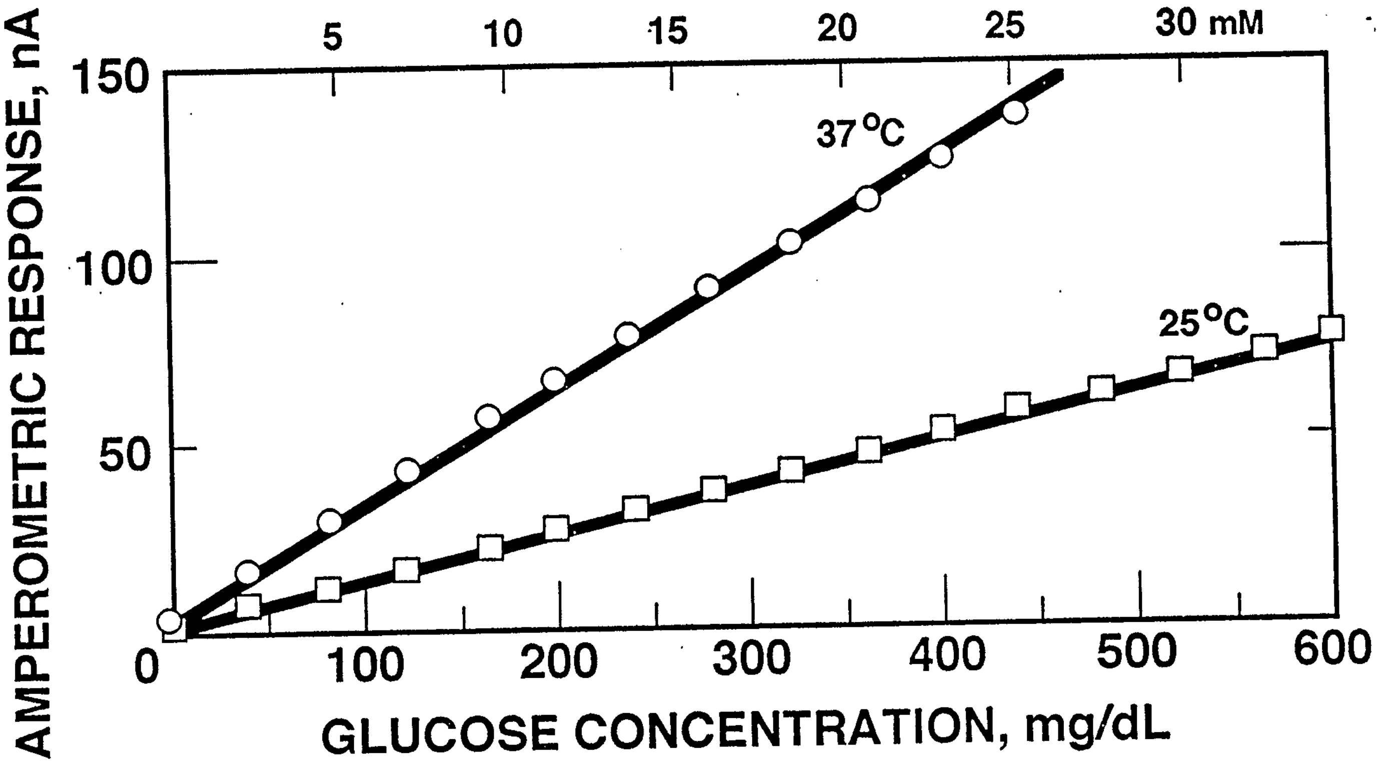


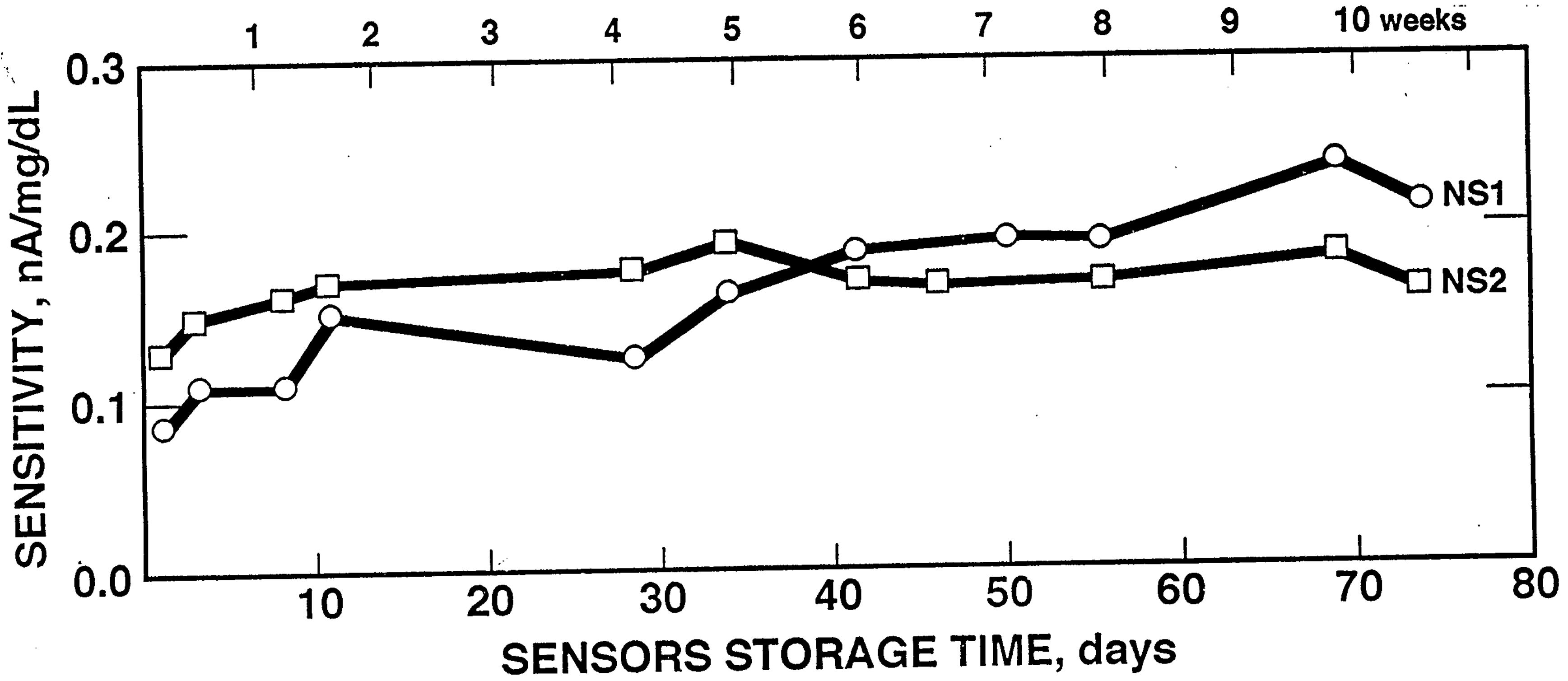
Q.Yang et al., FIG. 2



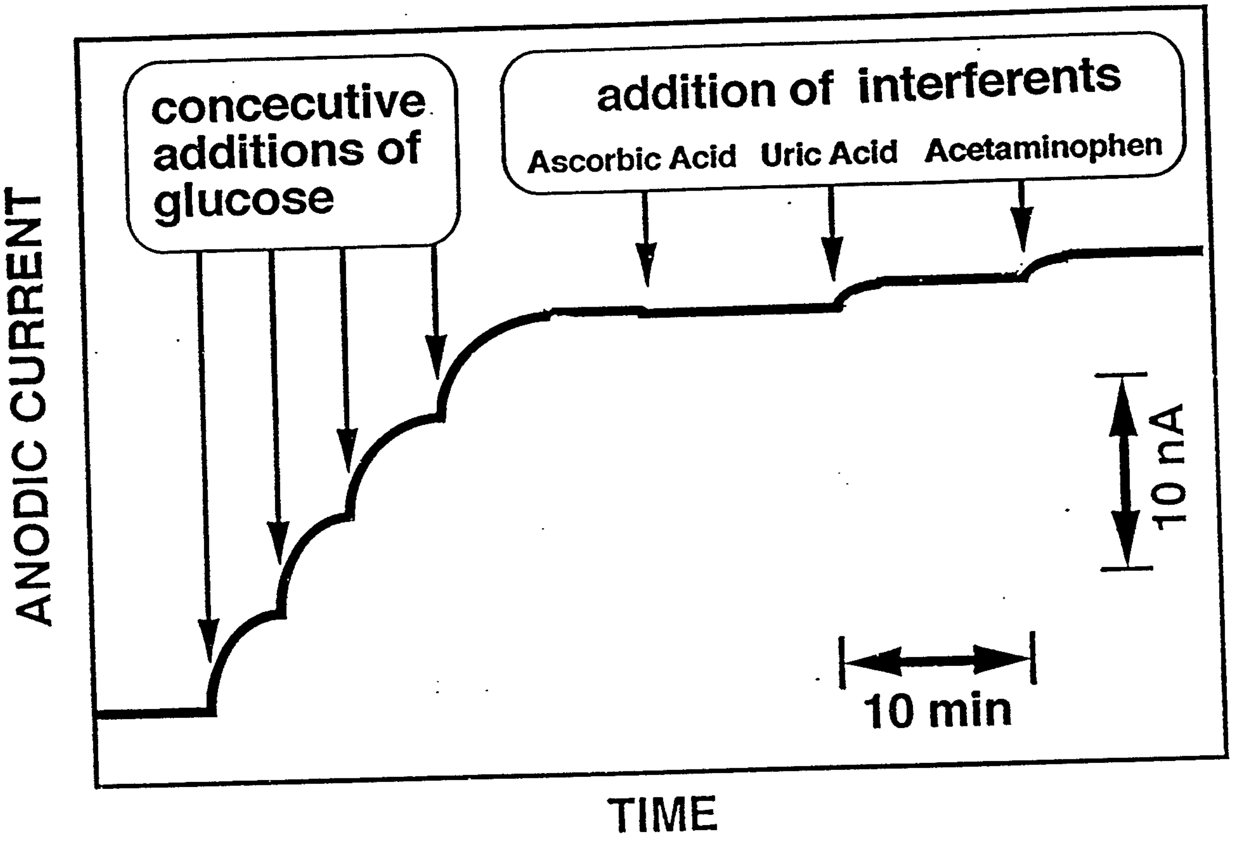
Q.Yang et al., FIG. 3



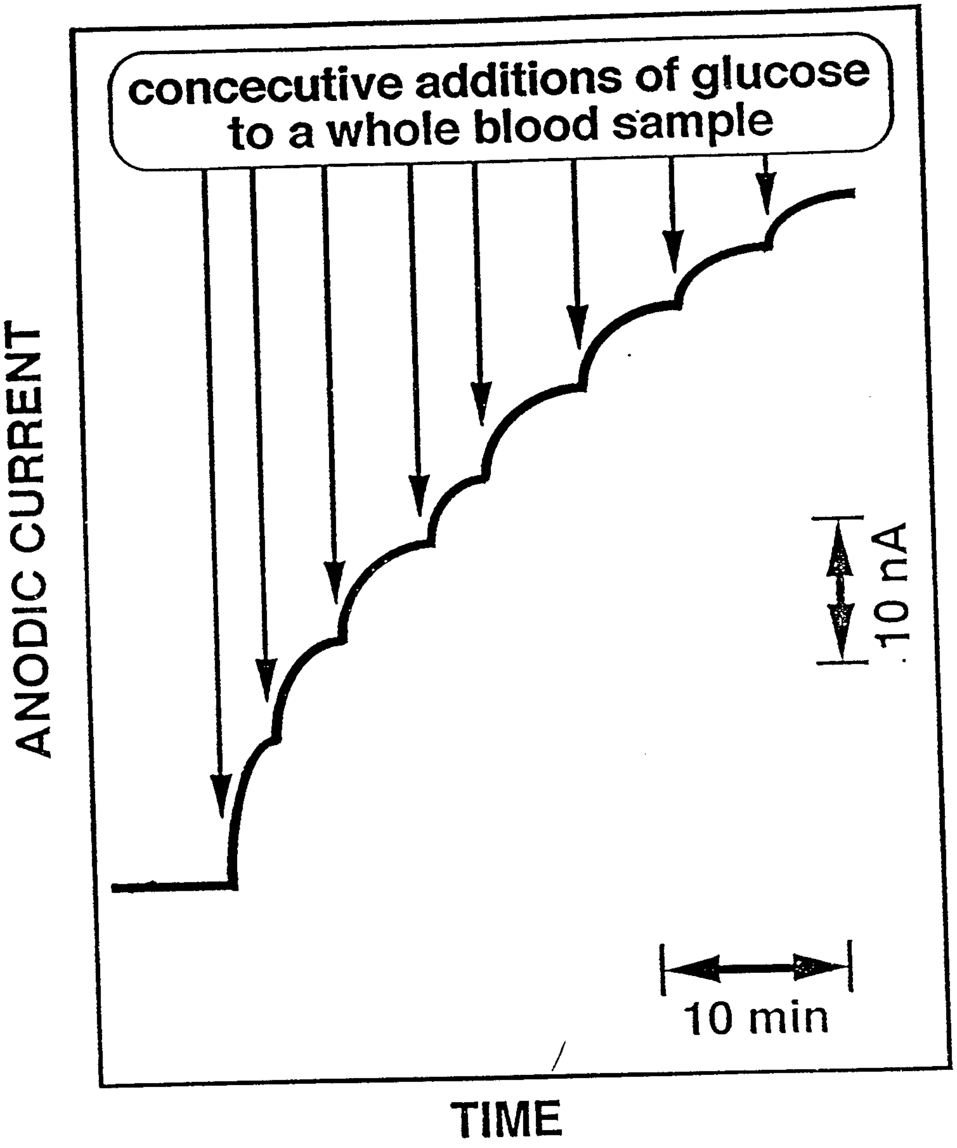
Q.Yang et al., FIG. 4



Q.Yang et al., FIG. 5



Q.Yang et al., FIG. 6



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Set Refs

Needle-Type Glucose Biosensors Based on a Pyrolyzed Cobalt-Tetramethoxy-Phenylporphyrin Catalytic Electrode

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APPENDIX 5

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Abstract
Amperometric needle-type glucose biosensors employing nonplatinum catalytic electrodes made from pyrolytic electrode of pyrolyzed cobalt-tetramethoxy-phenylporphyrin (CoTMPP) are described. A technique for preparation of the working electrode with catalyst and adsorbed enzyme (enzyme electrode) from a pressed matrix (tablet) is presented. This technique allows manufacturing numerous sensors from the same pressed tablet, all of which demonstrate a good reproducibility of their parameters (sensitivity, linear range, lifetime). Different glucose diffusion (external) membranes have been studied in order to extend the linear range of the biosensor response. Linearity of the sensor response to glucose concentrations up to 35 mM is reported when a cellulose paper membrane, impregnated with cellulose acetate, is used pressed into the needle sensor body. Sensors demonstrated a shelf lifetime of 30 days (with constant parameters of the signal output) and can be used for continuous glucose monitoring in vitro for at least 5 days.

Keywords: Pyrolyzed cobalt-tetramethoxy-phenylporphyrin catalyst, Needle glucose biosensor

1. INTRODUCTION

The development of sensors for glucose concentration determination has been the focus of much research during the past decade [1]. The attention given to this area of research arises from the clinical importance of glucose level monitoring in physiological fluids, especially with respect to the management of diabetes [2]. Implantable glucose biosensors designed to monitor glucose concentration in interstitial fluid (subcutaneous sensors) and in whole blood (intravenous, intravascular sensors) are being studied intensively [3–7].

The main technique for the construction of these glucose sensors is the use of enzyme electrodes [3]. This is because of the high substrate specificity and high activity of enzymes as biological catalysts. The enzyme usually used is glucose oxidase (GOD), and the detection of glucose is based on the following overall reaction:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{GOD}} \text{Gluconic acid} + H_2O_2 \quad (1)$$

Amperometric glucose biosensors are usually based on measuring the increase in the anodic current due to H_2O_2 oxidation, or the decrease in the cathodic current due to O_2 reduction. The H_2O_2 electrode based glucose biosensors are advantageous because they can be fabricated easily and constructed in small series even with conventional technology.

Needle-type sensors are one of the first approaches to design miniaturization of glucose sensors. Shichiri et al. [3] were the first to report success in miniaturizing a glucose sensor and introduced the needle enzyme electrode which had an outer diameter of 1 mm. Since then, there have been many reports on the development of needle-type sensors [4–7].

The needle sensor generally consists of a platinum working electrode (polarized as anode) and silver [3], stainless steel [4], Ag/AgCl [8], or platinum [5] as counter electrode, used as a reference in a two electrode system. A three electrode system with an Ag/AgCl as a wire reference electrode placed inside the needle body has also been reported [9].

In the last few years the use of different types of N_4-metal chelates (phtalocyanines, phorphyrins) for electroanalytical and biosensor applications has been the subject of many publications [10–22]. These investigations have been summarized in recent reviews [21, 22] presenting the electroanalytical applications of such catalyst and electrode modifiers as a dynamic area of research. The use of N_4-metal chelates offers several advantages: they show high catalytic activity combined with some analyte selectivity; they are suitable for forming thin layers over an inert electrode; they demonstrate compatibility with enzymes and different immobilization reagents. Oxygen reduction as well as red-ox properties of numerous organic compounds have been studied on electrodes modified with N_4-metal chelates [21, 22]. Some of N_4-metal chelates are employed as catalysts for electrochemical oxidation of hydrogen peroxide [15, 20].

It is known that the treatment of N_4-metal chelates in an inert gas at elevated temperature of 600 to 800 °C (pyrolyzation) increases their activity for electrochemical reduction of molecular oxygen as well as heterogenous decomposition of hydrogen peroxide ("catalase-like activity) [23–25]. Catalysts based on the pyrolyzed N_4-metal chelates are used mainly in gas diffusion electrodes for metal–air batteries and fuel cells [26].

In our earlier work it was shown that electrodes based on dispersed pyrolyzed cobalt-tetramethoxy-phenylporphyrin (CoTMPP) can be used as a hydrogen peroxide oxidation electrodes in biosensors for measurement of glucose and lactate [27, 28]. In these works a stationary catalytic hydrogen peroxide sensor was used in combination with different membranes with immobilized enzymes (glucose oxidase or lactate oxidase) mechanically attached to the electrode surface.

In this work, the performance of a needle-type glucose biosensor based on a pyrolyzed CoTMPP catalytic electrode is described. This article describes a prototype of a disposable miniature electrode with enzyme (glucose oxidase) immobilized directly on the surface of the pyrolyzed CoTMPP electrode as well as a new tablet based approach for mass fabrication of microsensors.

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2. Experimental

2.1. Reagents

Glucose oxidase (GOD, E.C.1.1.3.4, activity 250 EU.mg^{-1}, from *Aspergillus Niger*) was from Sigma Chemical Co.(St. Louis, MO), and was used without further purification. Cobalt-tetramethoxy-phenylporphyrin [CoTMPP, 5,10,15,20-tetrakis(4-methoxy-phenyl) -21*H*,23*H*-porphine cobalt(III)] was from Aldrich Chem. Co. (Milwaukee, WI). The Nafion perfluorinated ionomer was also obtained from Aldrich as 5 wt.% solution and used after dilution to 0.5% using 0.1 M phosphate buffer solution, containing 0.1 M KCl (pH 7.4). Stainless steel 14 and 22 gauge needles (outer diameter 1.27 mm) manufactured by Becton Dickinson Standard & Co. (Rutherford, NJ) were used. D-glucose, anhydrous (Dextrose, Baker Analyzed, Phillipsburg, NJ) was used as a stock solution (20 g/L) in phosphate buffer, prepared at least one day before measurements for mutarotation. The polymer coatings were: cellulose acetate (CA) from Eastman Kodak Co.(Rochester, NY), polyurethane SG80A (PU) from Thermedics Inc.(Woburn, MA) and polyvinylchloride 140 × 31 (PVC) was from BF Goodrich Co. (Avon Lake, OH). All other reagents used were of analytical grade.

Dispersed catalyst from pyrolyzed CoTMPP was prepared as described earlier [27, 28]. Crystalline CoTMPP was precipitated on a dispersed carrier (for example, carbon black) from DMFA solution in 1:1 weight ratio. After drying the material was pyrolyzed in argon at 700 °C for 4 h, and then cooled to room temperature in an argon stream overnight. The resulting black powder wass used as a disperse catalyst in the electrode preparation.

2.2. Enzyme Electrode Preparation

The catalytic electrode was fabricated in the form of a pressed tablet, from which a disk-shaped segment was placed inside a flattened needle tip. The preparation procedure is illustrated in Figure 1. This procedure consists of four consecutive steps: a) formation of a carrier tablet from Teflonized (PTFE treated) carbon black, b) formation of the catalytic layer over the pressed carbon black matrix, c) enzyme deposition (immobilization) on the catalytic layer and d) cutting a cylindrical segment from the tablet to obtain the enzyme electrode. The carrier layer is made of Teflonized carbon black [29] pressed at 260 KPa to give a electro-conductive matrix (Fig. 1a). The matrix has a disk shape with 10 cm^2 area and thickness about 0.4 mm. After the formation of the carbon black matrix, the dispersed catalyst from pyrolyzed CoTMPP is poured on one of the matrix sides (ca. 0.5 mg/cm^2) and pressed at 65 KPa to form a thin layer (Fig. 1b). The total thickness of the tablet after adding the catalytic layer does not exceed 0.5 mm. The resulting tablet is sufficiently elastic and durable to be stored or transported.

In general, electrodes of different shapes and sizes can be cut out from such a tablet. Macroelectrodes (disk-shaped, rectangular pieces or strips of several cm^2) as well aswellas microelectrodes (usually disks with diametersdimeters between 0.5 mm and 1 mm) can be fabricated. The reproducibility of this techniquetechique is high and relies on the reproducible properties of the Teflonized carbon black developed initially for the needs of the battery technology [29].

The side of the tablet with the supported pyrolyzed CoTMPP (catalytic layer) is impregnated with enzyme solution containing 20 mg/cm^3 GOD in 0.5% (w./v.) Nafion in phosphate buffer solution (Figure 1c). The enzyme–Nafion layer is then dried at room temperature for 2 h forming an active layer of immobilized GOD. The prepared enzyme layer contained approximately 1 mg/cm^2 GOD and ca. 0.25 mg/cm^2 Nafion (loaded amount) in the immobilization matrix.

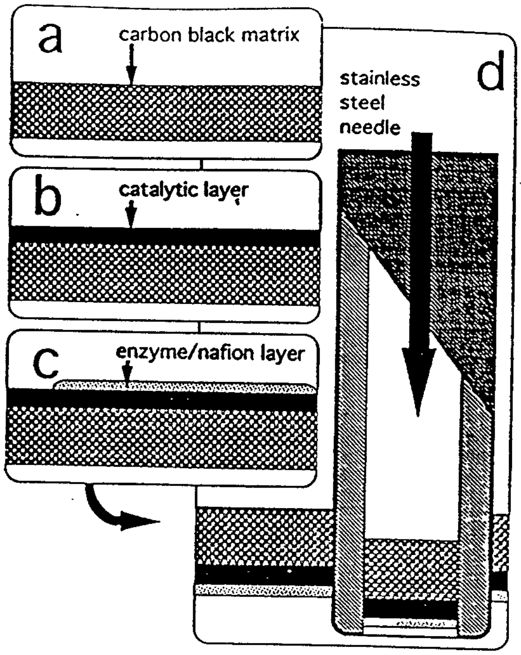

Fig. 1. Schematic of the processes of preparation of the needle-type enzyme electrode with pyrolyzed CoTMPP catalyst: a) formation of the tablet from Teflonized carbon black; b) formation of the catalytic layer of pyrolyzed CoTMPP; c) formation of the enzyme/Nafion layer over the catalytic layer; d) cutting a segment from the tablet by a hypodermic needle.

A smoothed end of a 22 gauge needle was used to cut out a cylindrical segment from the three layer tablet (Fig. 1d). In this case the cutting is performed starting from the back (carbon black matrix) side of the tablet so the enzyme/Nafion layer over the catalytic layer remain at the face side of the enzyme electrode so formed.

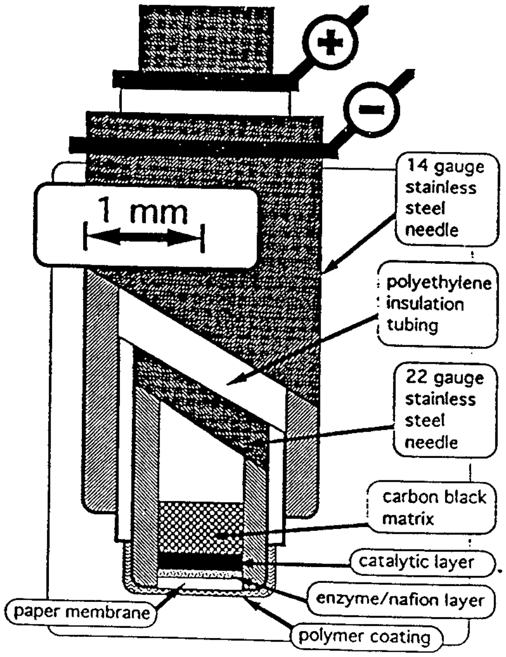
+
−
1 mm
14 gauge stainless steel needle
polyethylene insulation tubing
22 gauge stainless steel needle
carbon black matrix
catalytic layer
enzyme/nafion layer
paper membrane
polymer coating

Fig. 2. Crossectional schematic of the glucose biosensor based on the needle-type enzyme electrode with pyrolyzed CoTMPP catalyst.

2.3. Sensor Design and Preparation

A schematic of the glucose needle sensor crossection is shown in Figure 2. Two hypodermic needles are used in the sensor construction. The 22 gauge needle (with the cylindrical tablet segment) acts as body and current collector of the working electrode polarized as anode. The 22 gauge needle is insulated by polyethylene tubing and inserted into a tube cut out from another needle with larger diameter (14 gauge). This second needle serves as a counter electrode polarized as cathode.

Different polymer coatings are studied as a glucose diffusion membrane and sensor protection membrane. These coatings are made by dipping of the sensor tip in a polymer solution (5 % w./v. of CA in acetone, PU and PVC in tetrahydrofuran). The sensors were dipped in the solutions for 5 s and dried in air at room temperature for 15 min.

In the case where a cellulose paper membrane is used the paper segment is cut out from a plain sheet of white Xerox printer/copy paper during the enzyme electrode preparation [process d) on Fig. 1]. The paper piece is then impregnated with 2 % w./v. of CA solution in acetone).

2.4. Apparatus and Procedure

The needle sensors were tested in vitro in a magnetically stirred, thermostated measuring cell (volume 50 mL). A two electrode circuit was used, the catalytic working electrode polarized as an anode, and the stainless steel needle body as both reference and counter electrodes, polarized as a cathode, and maintained by a potentiostat (BAS CV-1B, Bioanalytical Systems Inc., West Lafayette, IN). The amperometric signals were recorded on Omnigraphic 100 recorders (Houston Instruments, Austin, TX). The supporting electrolyte was 0.1 M phosphate buffer solution, pH 7.4 containing 0.1 M KCl. Between measurements the sensors were stored soaked in phosphate buffer (pH 7.4) in closed vessels at 4 °C. Before measurements the sensors were polarized for at least 10 min to establish the background current.

Calibration plots – the dependence of the steady state anodic current on glucose concentration – were obtained when the glucose concentration was varied by consecutive additions of glucose from stock solution to the measuring cell in 2.2 mM (40 mg/dL) glucose concentration increments.

Reproducibility of the sensor response was tested by recording the current signal to alternatingly varying glucose concentrations between 2.8 mM and 5.6 mM (50 mg/dL and 100 mg/dL), and between 5.6 mM and 11.2 mM (100 mg/dL and 200 mg/dL) glucose. Continuous monitoring of glucose in such conditions was used in the evaluation of the sensors operational life-time.

The storage life-time of the sensors was studied by periodically obtaining calibration plots for sensors kept at 4 °C between the measurements.

3. Results and Discussion

Steady-state polarization curves were obtained in order to estimate a working potential for the prepared needle sensors. Figure 3a shows a typical polarization curve of a needle sensor with pyrolyzed CoTMPP working electrode in the presence (6.7 mM, Fig. 3a, curve 1) and the absence (Fig. 3a, curve 2) of glucose in the cell. In glucose solutions a significant increase of the anodic current at potentials higher than +0.3 V occurs. The anodic current reaches an observable plateau in the potential range from +0.5 to +0.7 V. At more positive potentials the anodic current in the presence of glucose (Fig. 3a, curve 1) increases further due to the increase of the background current at these potentials (Fig. 3a, curve 2).

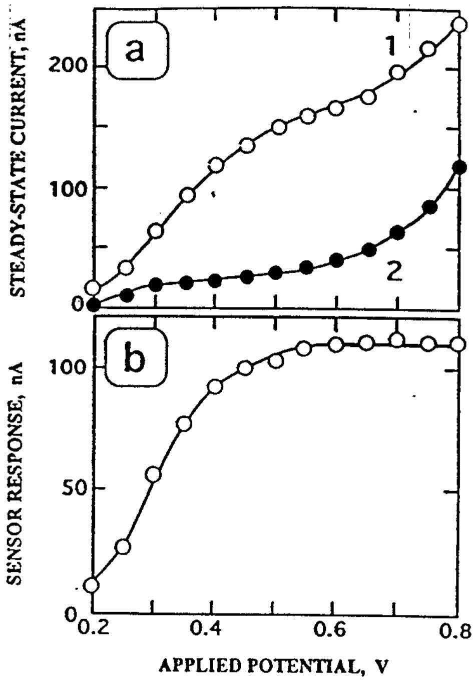

Fig. 3. Steady-state polarization curves of the pyrolyzed CoTMPP catalytic electrode vs. stainless steel needle, both incorporated into a biosensor: a) in 6.7 mM glucose solution (curve 1) and the background curve in blank phosphate buffer solution (curve 2); b) resulting polarization curve of the sensor response (subtraction of curve 2 from curve 1).

The difference between the anodic current in glucose solutions and the background current (this difference gives the sensor response) is plotted against the applied potential (shown in Fig. 3b). It is clear that a plateau is reached at potentials more anodic than +0.5 V forming the range of diffusionally limited electrooxidation of hydrogen peroxide at the pyrolyzed CoTMPP catalytic electrode. A working potential of +0.6 V was chosen, and all sensors were polarized at this potential.

3.1. Reproducibility of the Sensors Calibration Plots

The described technique of manufacturing the working electrodes from a preliminary pressed tablet allows the preparation of numerous sensors from a single matrix. Several series of sensors have been made out of different tablets. Results presented in this study are obtained with glucose biosensors using an enzyme – CoTMPP – carbon black matrix cut out from one tablet (tablet 'N'). The total number of electrodes (and biosensors) made from this tablet was 83. Before further treatment – coating with polymer – long-term operation or storage life-time tests of the biosensors were carried out obtaining their response to glucose by means of calibration plots.

Calibration plots of six needle sensors with catalytic electrodes from pyrolyzed CoTMPP, all from the described series, are shown in Figure 4. A linear dependance of the current response on glucose concentration is observed up to 16 mM glucose, and the sensitivity is from 10 to 12 nA/mM. Parameters of the calibration plots (linear range and sensor sensitivity) of all the

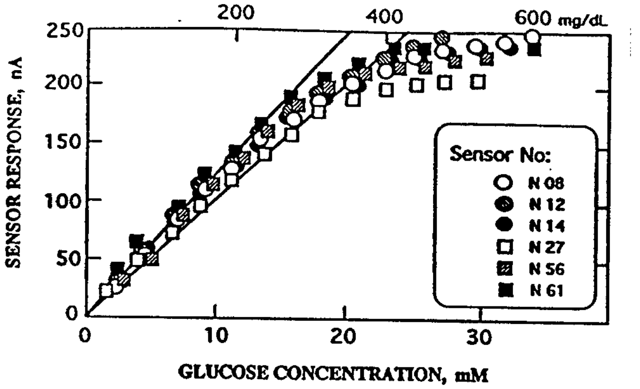

Fig. 4. Calibration plots of the biosensors in glucose buffer solution obtained with noncoated pyrolyzed CoTMPP catalytic electrodes prepared from one and the same tablet (randomly chosen set of six biosensors out of 83 prepared from the same tablet).

sensors prepared from the same tablet are similar. The upper limit of the linear range is between 13–16 mM glucose. The mean value of the sensors sensitivity is 11.3 nA/mM ± 1.2 nA/mM ($n = 83$). These results demonstrate the reproducibility of the manufacturing technique developed and provide a basis to compare characteristics of sensors made from one tablet.

3.2. Calibration Plots of the Sensors with Different Polymer Coatings

Several polymer coatings were investigated in order to extend the linear range of the sensors calibration plots: polyurethane (PU), polyvinylchloride (PVC) and cellulose acetate (CA) were all employed. Calibration plots were obtained for both noncoated and polymer-coated sensors. Fig. 5a shows examples of calibration plots obtained with a noncoated sensor (Fig. 5a, curve 1), a sensor coated with CA (Fig. 5a, curve 2), with PVC (Fig. 5a, curve 3) and with PU (Fig. 5a, curve 4).

Parameters of the sensors calibration plots – linear range and sensitivity – were the focus of this investigation. Analysis of these two parameters of the sensor response is appropriate using the electrochemical Eadie–Hofstee coordinates: response vs. sensitivity. This plot is based on the linearization of Michaelis–Menten equation when reaction rates are substituted with steady-state current fluxes:

$$I_S = I_{S_{max}} - K_{M^{app}}(I_S/C_g) \qquad (2)$$

Here I_S is the steady-state current of the electrode (amperometric signal), C_g is the concentration of glucose [so that (I_S/C_g) gives the sensitivity], $K_{M^{app}}$ is the apparent Michaelis–Menten constant of the enzymatic reaction (Eq. 1), and $I_{S_{max}}$ is the intercept on the current axis.

It can be seen from Equation 2 that in the case when the enzymatic reaction is the rate controlling process (kinetic control) the response–sensitivity dependence is linear with negative slope $K_{M^{app}}$. This means that the response–concentration dependence is hyperbolic (not linear in any concentration range).

Diffusion limitation of the overall processes occurring on the electrode resulted in a strong linearity of the signal–concentration dependences (calibration plots) following the equation:

$$I_S = nFAD^{eff}(C_g - C_o)/L_m \qquad (3)$$

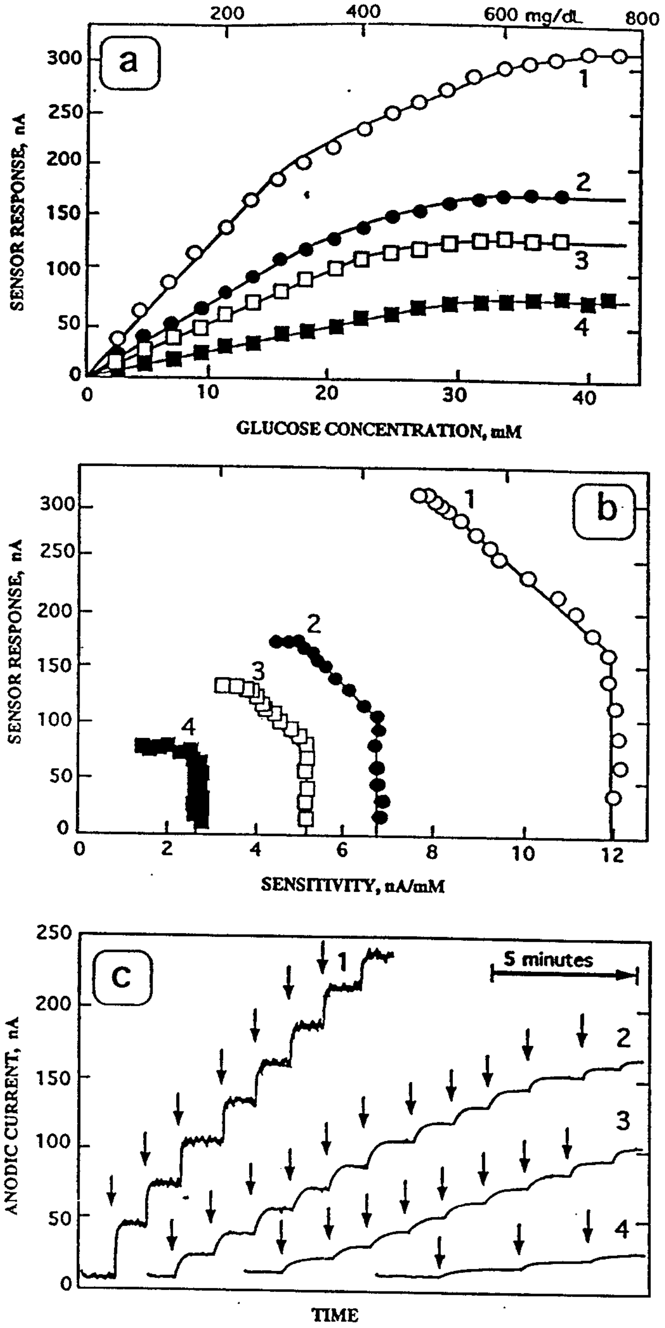

Fig. 5. Amperometric response to glucose of biosensors based on pyrolyzed CoTMPP catalytic electrode employing different polymer coatings as external membrane: 1) noncoated sensor; 2) sensor coated with CA; 3) sensor coated with PVC; 4) sensor coated with PU.
a) calibration plots of the biosensor in glucose buffer solution; b) Eadie-Hofstee plot of the data from (a); c) protocols of the biosensors response to consecutive additions of glucose (moments of the glucose addition are shown by arrows).

Here n is the number of electrons exchanged, A is the electrode surface area, D^{eff} is the effective diffusion coefficient of the substrate in the membrane, L_m is the membrane thickness, C_g is the bulk substrate concentration and C_o is the surface substrate concentration. When the electrode polarization is sufficiently high and the enzymatic reaction is fast compared to diffusion (diffusion control), then $C_o = 0$ and the amperometric response is directly proportional to the substrate (glucose) concentration.

In Eadie–Hofstee coordinates this dependence is shown by a vertical line parallel to the current axis. Most real cases are characterized by mixed diffusion and kinetic control of the processes.

Presentation of the experimental data from Fig. 5a in Eadie–Hofstee coordinates is given in Fig. 5b. It can be seen that the curves obtained with the noncoated sensor (Fig. 5b, curve 1) as well as those obtained with sensors coated with CA (Fig. 5b, curve 2), PVC (Fig. 5b, curve 3) and PU (Fig. 5b, curve 4) are complex and consist of at least two parts: the first part has a portion with constant sensitivity (vertical lines) and a linear portion with a negative slope. These plots could be interpreted as an evidence of a mixed control, predominantly diffusion at low glucose concentration and kinetic control at higher glucose concentration. The part of the calibration plots from Fig. 5a which corresponds to the vertical portion of the Eadie–Hofstee plot of the same data in Fig. 5b presents the linear range of the sensor response to glucose.

The biosensor with no external polymer layer shows a linear dependence of the response to glucose concentration up to 13 mM. The sensor with CA coating demonstrates linear response up to 16 mM glucose, while the PVC coated sensor has a linear range up to 22 mM glucose. The largest linear range obtained with a single polymer coating is in the case of PU membrane, up to 27 mM glucose. The linear dependence of the sensor response to glucose concentration in case of an noncoateduncoated sensor is probably due to the properties of the enzyme–Nafion immobilization matrix, which acts as a diffusion membrane. An additional contribution to this might be provided by the roughness of the tablet surface. Polymer coating over the enzyme–Nafion layer act as a glucose diffusion membrane which results in the increase of the linear range of the sensor response.

It can be seen from Fig. 5a and b that the increase in the linear range of the sensors response is associated with a decrease in the sensor sensitivity. Sensitivity of the noncoated sensor is approximately 12 nA/mM. Sensors with CA coating demonstrate a slope of the linear portion of the calibration plot of 6.7 nA/mM. Sensitivity of the PVC-coated sensor is 5.2 nA/mM and for the PU coated sensor sensitivity is 2.7 nA/mM.

In all cases, however, except for the PU-coated sensor, the linear portion of the Eadie–Hofstee plots have the same slope, demonstrating an average K_{Mapp} of 19.35 mM ±1.83 mM (standard deviation). The value of the K_{Mapp} remains unaffected by the polymer coating suggesting that it is a characteristic of the enzyme deposited (adsorbed) on the catalytic layer in the Nafion matrix.

In the case of PU polymer coating (Fig. 5a, curve 4) the sensor signal depends linearly on glucose concentration up to its level of saturation. This level, however, is far lower (by a factor of 5) than the saturation level of the noncoated sensor (Fig. 5a, curve 1). This could be a result of some partial loss of enzyme activity during the treatment of the sensor with THF polymer solution, or it might be due to transport hindrances for glucose in the PU membrane.

The response time (estimated as time to reach 95% of the steady-state value of the current signal) is also affected by use of different polymer coatings. Fig. 5c presents typical protocols of the current transients for the four sensors (from Fig. 5a) during the calibration test. It can be seen that the response time of the sensor does not depend on concentration when the sensor operates within its linear range. The response time in this case is a function of the concentration step-change only. The response time (for a concentration step-change of 2.2 mM) for the noncoated sensor is 5–10 s. (Fig. 5c, curve 1). For the sensor with CA coating it is approximately 15 s. (Fig. 5c, curve 2) and for the sensor coated with PVC this time is about 30 s. (Fig. 5c, curve 3). A glucose sensor coated with a PU polymer layer demonstrates a response time of up to 2 min (Fig. 5c, curve 4). At the highest glucose concentrations, when kinetics become rate limiting factor, the response time increases with increasing concentration of glucose.

3.3. Sensors with Cellulose Paper Membrane

A cellulose paper membrane was incorporated into a sensor body during the preparation procedure (Fig. 1, process d). The sensor with a mechanically attached (pressed in) paper membrane was tested and the obtained calibration plot was compared with a that of a sensor without membrane or polymer coating. The sensor with cellulose paper membrane demonstrated linear dependence of the amperometric response vs analyte concentration up to approximatelyapproximatelly 30 mM glucose. The value of the of the saturated current signal (at high glucose concentrations) obtained with the sensor with cellulose paper membrane is close to that obtained with a sensor with no external membrane (noncoateduncoated electrode). In further measurements a series of sensors employing cellulose paper membranes were studied ($n = 6$). The membrane was additionally impregnated by diluted CA solution in acetone (2 % w./v.) by dipping the already pressed assembly in the polymer solution.

Figure 6 presents the calibration plots of a glucose biosensor from this series before (Fig. 6, curve 1) and after attaching a cellulose paper membrane (Fig. 6, curve 2). It can be seen from the figure that the sensor with the additional cellulose paper membrane has a linear range of amperometric response to glucose up to 33 mM. The values of the steady-state current at signal saturation (at glucose concentration higher than 40 mM) obtained with and without cellulose paper membrane are practically identical. Linear regression equation of the experimental data (Fig. 6, curve 2) can be expressed as:

$$I_S[\mathrm{nA}] = (0.4 \pm 0.9)[\mathrm{nA}] + (7.35 \pm 0.27)[\mathrm{nA/mM}] \times C_g[\mathrm{mM}] \tag{4}$$

The correlation coefficient of the linear regression (R^2) is 0.998; $n = 15$ data points were used, which gives an upper limit of the linear range as 33 mM.

Response time of the biosensors with cellulose paper membranes (when the glucose concentration step-change is 2.2 mM) is about 20 s. and was independent of the glucose concentration over the entire range of investigated glucose

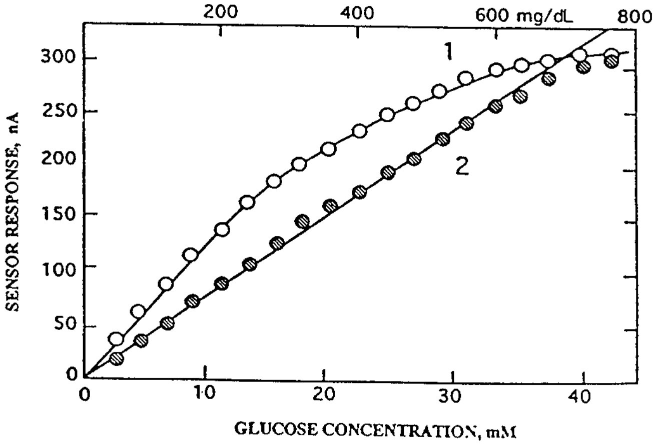

Fig. 6. Calibration plots of the biosensor response to consecutive additions of glucose for the noncoated biosensor (1) and for the same biosensor after incorporating a cellulose paper membrane (impregnated with CA) in the enzyme electrode (2).

concentrations (up to 45 mM). Value of the amperometric response of the sensor to glucose (the anodic current) does not depend on the speed of stirring (agitation in the measuring cell) and coincides with the current response obtained in non-agitated samples. Hence boundary–layer effects do not affect the sensor response.

The cellulose paper membrane acts as a matrix for the electrolyte immobilization. It provides sufficient diffusion limitation to the analyte flux, avoiding the blocking effect of the polymer coatings. Immobilization of the solution causes signal independence from agitation. As a result this type of sensors demonstrates the largest upper limit of the linear range combined with sufficient sensitivity and low response time.

3.4. Reproducibility of the Sensor Response and Life-Time Performance

The reproducibility of the response of the sensors was tested by varying the glucose concentration in the measuring cell alternatively between two levels of glucose concentration with step-changes of 2.8 mM between 2.8 mM and 5.6 mM, and with step changes of 5.6 mM between 5.6 mM and 11.2 mM. The sensors monitored the glucose level in the cell for 2 h between concentration changes. Continuous testing for at least five days was performed; overnight sensors were left operating at the lowest glucose concentrations. It is found that for every level of glucose concentration, the sensor response attained the same amperometric signal value within an acceptable ± 2.5 % error limit. The response time of each type of the investigated sensors at the concentration step-change of 5.6 mM is approximately 3.5 times higher than that estimated with the concentration step-change of 2.2 mM during the calibration tests (see Fig. 5c). There is no observable difference between the response times measured for increasing concentration, or for decreasing concentration.

During the described reproducibility tests the sensor operation life-time was estimated. Sensors were used in continuous monitoring of glucose concentration ranging from 2.8 to 11.2 mM (which covers the interval of physiological glucose levels) for approximately one week, and they demonstrated no significant decrease of the sensor signal. During the second week of continuous operation, the signal of all types of sensors began to decrease and the tests were terminated when the signal value reached 30 % of the initial value.

The storage (shelf) life-time of the sensors (at 4 °C) was studied by periodically obtaining sensor calibration plots. Figure 7 presents the dependence of the sensor sensitivity on the storage time for all types of the sensors investigated. Data presented are the average values of the slopes of the linear portion of the calibration plots for four independently tested sensors from each type. The starting point of the time axis is the day of sensor fabrication from the tablet. Between the calibration plot measurements the sensors were kept (stored) in 0.1 M phosphate buffer solution at 4 °C. The results presented in Figure 7 show that the sensitivity of the sensors remain practically constant for up to one month of storage (within 10 % to 15 % of the initial value) for all types of sensors. The decrease of the sensor sensitivity during the further storage is probably caused by either the loss of enzyme activity or desorption of GOD from the enzyme–Nafion layer.

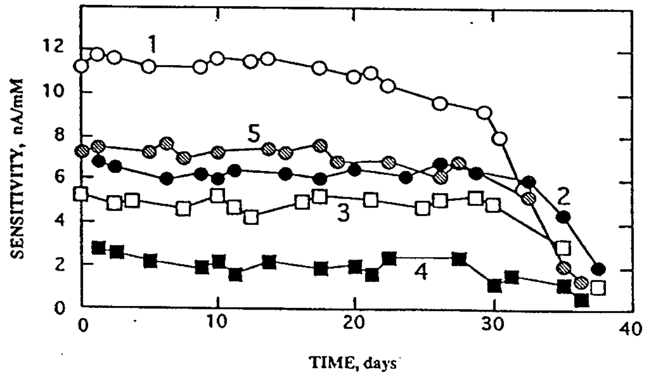

Fig. 7. Dependence of the sensor sensitivity vs. time of the sensor storage in blank phosphate buffer at 4 °C for: 1) noncoated sensor; 2) sensor coated with CA; 3) sensor coated with PVC; 4) sensor coated with PU; 5) sensor with cellulose paper membrane impregnated with CA.

4. Conclusion

The results presented show that a nonplatinum catalytic hydrogen peroxide oxidizing electrode can be used successfully in a needle-type glucose biosensor. The proposed technique for enzyme electrode preparation from a pressed multilayer carbon black tablet provides the possibility to improve the reproducibility of the sensor parameters within a set of sensors. By employing different polymer coatings, the linear range of the sensor response to glucose is extended up to 27 mM glucose (PU coating). The use of a cellulose paper membrane extended the linear range of sensors up to 33 mM without noticeable decrease in sensor sensitivity and signal amplitude. The sensors demonstrate stable signal response for up to 5 days of continuous monitoring in phosphate buffer within the physiological concentration range of glucose. Shelf life-time of the sensors is estimated to be about one month when stored in phosphate buffer at 4 °C.

5. Acknowledgements

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ELECTROANALYSIS

VCH

February 13, 1995

Dear Professor Wilkins:

The Manuscript entitled "Needle-type glucose biosensors based on a pyrolyzed cobalt-tetramethoxy-phenylporphyrin catalytic electrode" (EL94-197)

has been accepted for publication and will appear in an early issue.

Very best regards.

Sincerely,

J. W

Joseph Wang
Editor-in-Chief

PS: Please stop by during the April meeting!

ANALYTICAL LETTERS, 28(14), 2439-2457 (1995)

ENZYME ELECTRODES WITH GLUCOSE OXIDASE IMMOBILIZED ON STÖBER GLASS BEADS

KEY WORDS: Sol-gel matrix, Stöber glass beads, Glucose enzyme electrode

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ABSTRACT

In this study we report the development and experimental evaluation of a miniature Stöber glass-coated enzyme electrode for glucose biosensor applications. The enzyme - glucose oxidase - is chemically immobilized onto the surface of the Stöber glass beads after a silanization procedure. The sensors were fabricated using glass beads of different radii ranging from 20 nm to 100 nm. The overall thickness of the glass coating was about 1.4 μm in all cases. Initial calibration curves of the electrodes show an increase in the response value with decrease in the bead radius. Life-time evaluation tests, however, demonstrate a different behavior of the electrodes. At the end of a 50 day test, the electrode with bead radius of 70 nm remained most stable. The response of this electrode was stable over this period of time and no apparent decrease in its sensitivity was observed. This study suggests that there may be a relation between the immobilized enzyme stability and the

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porosity of the glass-bead layer. Preliminary tests in undiluted blood plasma suggest that electrodes can be used for glucose measurements in body fluids.

INTRODUCTION

The development of sensors for invasive glucose monitoring has been the focus of much research during the past decade. This attention arises from the clinical importance of these sensors, specially in the management of diabetes[1]. Amperometric enzyme electrodes present the most common approach because of the high substrate specificity and activity of enzyme, and the proportionality of the current signal to glucose concentration provided by amperometry. The oxidation of glucose by molecular oxygen to form gluconic acid and hydrogen peroxide,which is catalyzed by Glucose Oxidase (GOD), forms the basis of glucose detection.

Needle-type glucose sensors based on hydrogen peroxide electrodes have been the most attractive approach because of their ease of fabrication and adequate performance for short term implantation[2-4]. Success with this approach has reached the stage of implantation[5,6].

Several techniques have been developed for the immobilization of enzymes. Previous work focused mainly on organic matrixes[7], but now more and more attention is being paid to the inorganic materials, as such matrixes have a number of advantages over organic ones: resistance to microbial attack; resistance to change in swelling and porosity with pH, and the excellent storage stability of enzymes.

Sol-gel is a low-temperature technology for the production of ceramic materials through the formation of colloidal suspension of metal oxides[8]. This technology and the associated sol-gel doping technology provides an attractive method for immobilization of bio-molecules in the silica gel because of the low temperatures involved in the preparation. Avnir *et al*[9] demonstrated that it is possible to immobilize organic compounds in inorganic supports by introducing the organic compounds with the polymerization precursors. Indeed, this new class of organic-inorganic materials has found a plethora of diagnostic[10] and other applications[11]. Braun *et al*[12] demonstrated the possibility of protein immobilization in sol-gel silica matrices.

Enzyme immobilization on sol-gel silica supporting matrix can be by adsorption, entrapment, or cross-linking. Much of the work has used the physical entrapment method. Several leading research groups in this field[9,11,13,14] have used cyclic voltammetry to study the activity of GOD doped within a tetramethoxysilane (TMOS) - derived sol-gel matrix. In this scheme the sol-gel was doped with GOD and a mediator, hydroxymethyl ferrocene. This mixture was then coated onto the distal end of a glassy carbon electrode. Enzyme activity was estimated to be 70-80% in the fresh polymeric silica sol, but no information on the activity as the coating aged was provided. Glezer *et al* [15] reported a new class of enzyme electrodes made of conductive, porous vanadium pentoxide prepared by the sol-gel doping procedure. Cyclic voltammetry was used as the mode of detection for glucose. The metrological characteristics of the vanadium oxide biosensors such as the range and sensitivity of detection are similar to those of commercial glucose electrodes. Saturation of signal was observed beyond 10 mM ß-D-glucose. The calibration curve of the vanadium pentoxide biosensor was stable during prolonged storage, and the electrode did not lose any activity during 10 days of storage at 4°C. Tsionsky *et al*[16] introduced sol-gel-derived ceramic-carbon composite electrodes. These electrodes are rigid, porous, easily modified chemically and have a renewable external surface. The electrodes offer higher stability than carbon paste electrodes, and are more amenable to chemical modification than monolithic and (organic) carbon electrodes. Tatsu *et al*[17] prepared tetraethoxysilane (TEOS) - based sol-gel monoliths doped with GOD and reported on their performance as glucose-sensing elements. In a flow injection analysis scheme, the monolith-based sensor exhibited a peak response time on the order of 4 min. Saturation of the signal response was observed over 400 mg/dL, and the GOD activity was shown to vary over time, depending on the actual storage temperature. With increase in aging temperature, the yield increased but the activity decreased. The activity of the entrapped GOD was found to be 20-fold greater when stored at -20°C compared with room temperature. The sol-gel-encapsulated GOD was reported to remain active for at least 2 months when stored desiccated at 4°C.

Dvorak & Armond[18] described some examples of electrode modification by a sol-gel silicon dioxide gel film doped with trisruthenium (II) chloride, which retained its electrochemical activity and exhibited an anodic photoeffect. The comparison of photochemical and electrochemical data was used to estimate the active fraction of the encapsulated complex.

Braun *et al*[19] reported on the properties of a 8 x 2 mm disk of tetramethyl orthosilicate-derived xerogel doped with glucose oxidase, peroxidase, and a chromogenic dye for detection of glucose. This disk-based device did indeed respond to glucose, but information on enzyme activity, stability, detection limits, and response time were not presented.

The approach used in this study is based on exploiting chemical reaction between primary amino groups from the silanization process on the support material and the amino group on the enzyme. The two terminal aldehyde entities of glutaraldehyde cross-link the two amino groups on the support material and the enzyme. In this reaction water is eliminated and Schiff-base type of bonds ($R_iN=CHR_j$) are formed:

$$[\text{Support}]\text{-}NH_2 + OHC\text{-}(CH_2)_3\text{-}CHO \xrightarrow{-H_2O} [\text{Support}]\text{-}N{=}CH\text{-}(CH_2)_3\text{-}CHO$$

$$[\text{Support}]\text{-}N{=}CH\text{-}(CH_2)_3\text{-}CHO + H_2N\text{-}[\text{Enzyme}] \xrightarrow{-H_2O} [\text{Support}]\text{-}N{=}CH\text{-}(CH_2)_3\text{-}CH{=}N\text{-}[\text{Enzyme}]$$

In this way, the glutaraldehyde actually acts as a cross-linking agent which fastens the enzyme chemically to the solid support. The support can have inherent amino groups as in the case of Nylon, or have some added by suitable pretreatment. Thus, silanization with 3-amino-propyltriethoxysilane is often used for the glass bead surface preparations.

EXPERIMENTAL

Reagents and Materials

Glucose Oxidase (GOD, E.C.1.1.3.4, activity 250 $EU.mg^{-1}$, from *Aspergillus Niger* was from Sigma Chemical Co.(St. Louis, MO), and used without further purification. Platinum wires of 0.127 mm diameter were from Aldrich Chemical Co., Inc. (Milwaukee, WI). 3-Aminopropyltriethoxy silane , glutaraldehyde (25%) and ß-D(+)-Glucose were from Sigma Chemical Co., the glucose was used as a stock solution (20 g/L)in phosphate buffer. All other reagents used were of analytical grade. Distilled water was used in all the experiments.

Blood plasma was prepared by centrifugation of heparinized fresh bovine blood, obtained from a local slaughterhouse. Glucose concentration in the different plasma samples was measured by standard clinical methods using a B-Glucose Analyzer (HemoCue AB, Angelholm, Sweden).

Sensor Preparation

Platinum wires were coated with a layer of Stöber glass beads by sol-gel technology using the facilities of Sandia National Laboratories. The porous Stöber glass films were made by multiple dip coating of Pt wires according to a previously published method[20]. After each dip the wires were cured at 400°C in air. The monodisperse glass spheres were made by the Stöber method[21] and maintained in ethanol-water (pH 11) solutions. Films made in this manner have a very high free volume and porosity. The perceived advantages of using Stöber glass films for immobilizing bioactive molecules such as enzymes include the rigid structure of the films, strong adhesion to the sensor surface (both pH sensing surfaces like SiN and the Pt wires have been fabricated), and high potential diffusion constants for both reactants and products. Other films, both organic polymers and inorganic sol-gel matrices, enmesh the biomolecules in a gel where diffusion constants can be lowered by factors of up to 10^3. It is known from BET type porosity and surface area measurements that Stöber films made from different size spheres have a well defined and reproducible porosity of 37%, which is larger than one would expect from close packing of the spheres.

Table 1. gives the parameters of the platinum wires coated with different sizes of Stöber glass beads. The thickness of the coating layer of Stöber glass beads in every case is close to 1.4 μm. The diameter of the glass beads used in the experiments ranged from 20 nm to 100 nm.

Fig. 1 shows SEM photographs of the Stöber glass beads layer coated on platinum wires. From the Figure it can be seen that the bead distribution becomes more homogeneous as the glass bead diameter increases. The coatings with 20 nm and 45 nm Stöber glass beads on the platinum electrode surfaces demonstrate some cracks. The cracks on the electrode coated with 20 nm beads are larger than that observed on the electrode coated with 45 nm Stöber glass beads.

TABLE 1. Parameters of the glass beads coated on the platinum electrode

Diameter (nm)	Thickness (μm)	Number of Coats
20	1.34	6
45	1.84	5
70	1.36	5
100	1.48	9

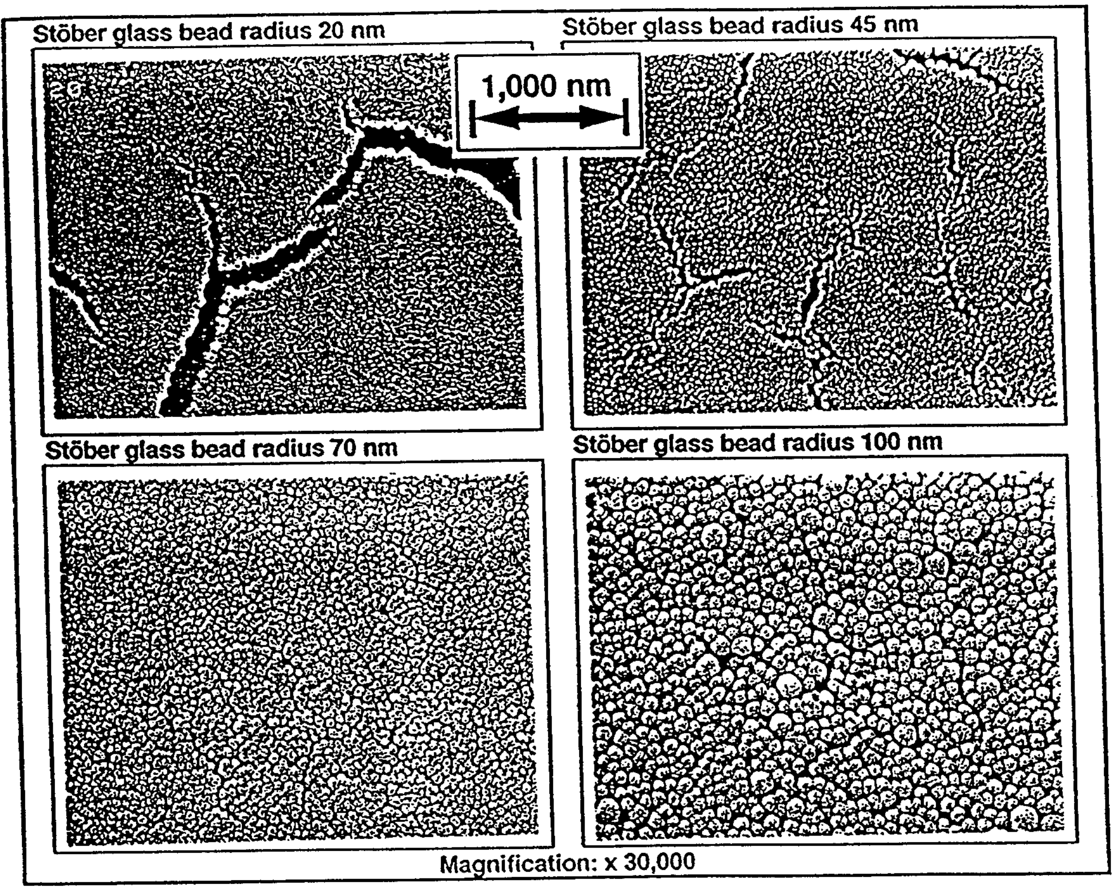
Stöber glass bead radius 20 nm
Stöber glass bead radius 45 nm
1,000 nm
Stöber glass bead radius 70 nm
Stöber glass bead radius 100 nm
Magnification: x 30,000

FIG. 1 SEM micrographs of the Stöber glass beads coated on the platinum electrode. Glass bead radius is listed on the right upper corner of each of the photographs

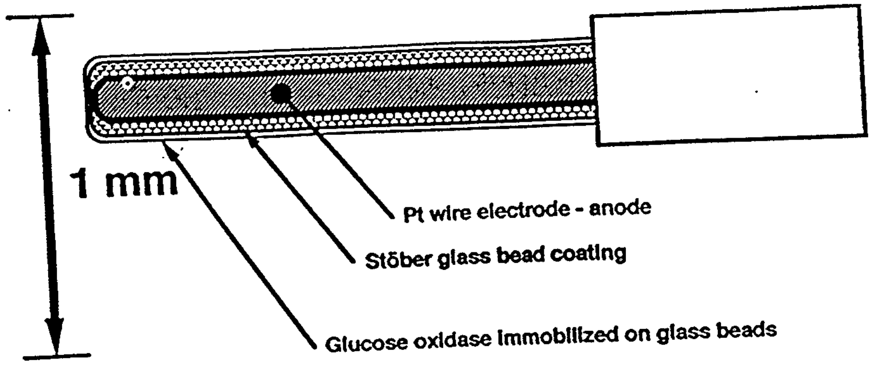
1 mm
Pt wire electrode - anode
Stöber glass bead coating
Glucose oxidase immobilized on glass beads

FIG. 2 Schematic of the glass-bead-coated glucose enzyme electrode

The cracks on the surface, if not eliminated, would result in a manufacturing process that would not yield identical biosensors. Thereafter, the bare platinum exposed via the cracks can oxidize other substances present in the medium and therefore increase interference effects. One of the sensors was a bare platinum wire and was used as a control.

Fig. 2 shows the schematic of the electrode construction. The electrode consists of a platinum wire, a Stöber glass beads coating layer on the platinum, and the outer immobilized enzyme layer. The glass coating layer has two effects: it acts as a support and matrix for the enzyme; it can be a barrier to some substances that may cause disturbance in the signal.

Silanization was utilized to prepare the Stöber glass beads and the platinum wire surfaces to bond with glutaraldehyde. Solution of 3-aminopropytriethoxy silane (0.2 mL) in distilled water (2 mL, to give a 10% v/v solution) was prepared. The pH was adjusted to 4.1 using hydrochloric acid (HCl) solution. This mixture was subsequently placed into a small vial and the five electrode immersed into the vial. They were kept in an oven at 80°C and shaken every fifteen minutes for three hours. The sensors were rinsed completely in distilled water and then dried at 120°C for three hours. The wires were stored in the refrigerator for 24 hours.

Immobilization was accomplished by first creating a layer of glutaraldehyde bonded to the silanized electrode surface. A 2.5% glutaraldehyde solution was made with 27 mL distilled water and 3 mL of 25% glutaraldehyde. Immobilization was accomplished by immersing the wire in the glutaraldehyde solution, continuously stirred, for one hour at room temperature. The wires were then rinsed with water for one hour at room temperature after that the electrodes were immersed in the enzyme solution. The enzyme solution was prepared with 80 mg of glucose oxidase in 2 mL phosphate buffer of pH 7.4. The wires were left in the enzyme solution overnight at 4°C to allow immobilization onto the glass layer.

Apparatus and Procedure

A three-electrode electrochemical cell was used to test the sensors. This system used a bare platinum electrode as a counter electrode, the glass coated enzyme electrode as a working electrode, and a silver/silver chloride electrode as a reference (standard) electrode.

Cyclic voltammetry and constant potential amperometry experiments were performed using a potentiostat (Bipotentiostat Model AFCBP1 Pine Instruments Co., Grove City, PA) which was connected to a recorder (Model 200 XY Recorder, The Recorder Company).

Experiments were carried out in a thermostated electrochemical cell with a volume of 25 mL. The concentration of solution in the cell was varied by consecutive additions of glucose. Phosphate buffer solution (pH 7.4) was used as a supporting electrolyte. During the experiments the solution in the cell was continuously agitated by a magnetic stirrer.

RESULT AND DISCUSSION

Cyclic voltammograms were obtained in order to estimate a working potential for the prepared electrodes. The cyclic voltammograms recorded at several glucose concentrations showed an increase of the current in the potential range of hydrogen peroxide oxidation. This indicates that the biosensing mechanism is *via* hydrogen peroxide oxidation (generated by enzyme oxygen reduction). In glucose solutions, a significant increase of the anodic current at potentials more positive than +0.35 V occurred. In these cases the anodic current reached an observable plateau at potentials more positive than +0.55 V. The current value in the plateau range increased with the increase of the glucose concentration. From the cyclic voltammetry studies a working potential of +0.6 V was chosen, and all sensors were operated polarized at this potential during the constant potential amperometry.

Performance of The Glucose Sensors

Fig. 3.a shows the calibration curves obtained initially after the immobilization process. From the Figure it can be seen that the signal depends on the size of the glass beads. The size of the glass beads affects the surface and pore distribution of the bead matrix and hence influences the amount of enzyme immobilized. The signal amplitude at given glucose concentration increases with decrease in the radius of the beads in the glass coating layer from 100 nm to 20 nm. Platinum wires which had no glass coating, but had the enzyme immobilized on the silanized surface responded with the largest signal. This is a result of the lack of diffusion-barrier for the diffusion of glucose and hydrogen peroxide to and from the electrode.

The response time of the electrodes (estimated as time to reach 95% of the steady state value of the sensor current signal) was about 1.5 min. This time was not affected by the radius of the glass beads in the coating layer.

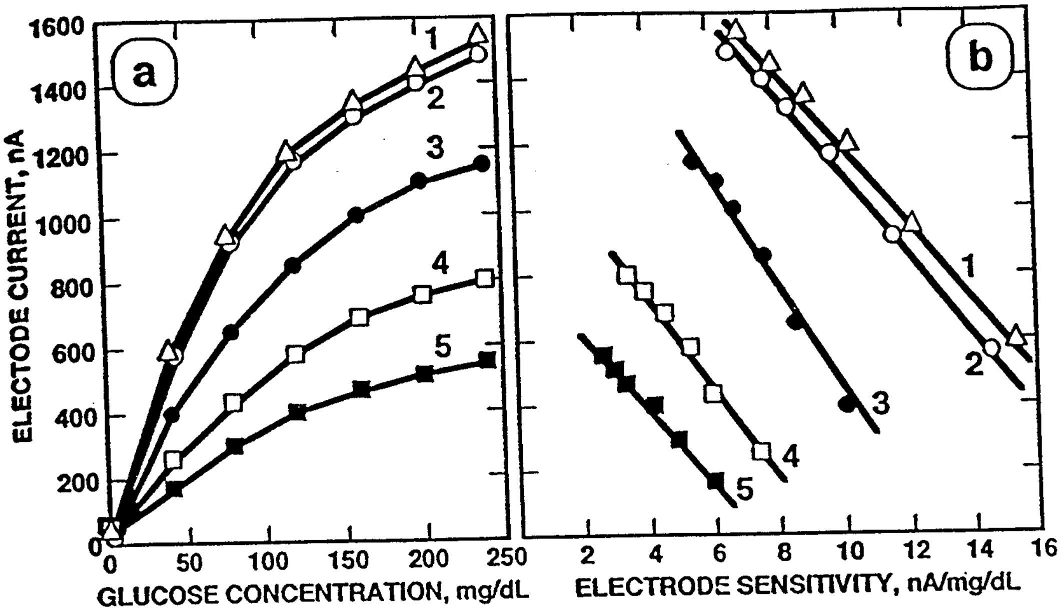

FIG. 3 (a) Calibration curves of the glucose enzyme electrodes coated with Stöber glass beads with different radius: non-coated electrode (1); bead radius of 20 nm (2); 45 nm (3); 70 nm (4); 100 nm (5). Curves correspond to the initial tests of each electrode.
(b) Eadie-Hofstee plots of the data presented on Fig. 3.a.

Fig. 3.b shows the data from Fig. 3.a in the Eadie-Hoftsee coordinates (electrode signal vs sensitivity). This plot is a linearization of the Michaelis-Menten equation and it can be used as an evaluation tool for enzyme electrode behavior. It can be seen from Fig. 3.b that the calibration curves data (from Fig. 3.a) are linearized to fit linear dependencies in the Eadie-Hoftsee coordinates characterized with negative slopes. From this slopes the apparent Michaelis constant of the electrode process can be calculated. The slopes of the Eadie-Hoftsee plot (Fig. 3.b) are almost the same. From the plot, an apparent Michaelis-Menten constant of 130 mg/dL is obtained. Since the external diffusion of glucose is fast compared to the rate of the reaction, the process was under the control of kinetics of the reaction.

The sol-gel process[22] offers many strategies for the preparation of inorganic materials with pore sizes in the range appropriate for membranes and sensors, and

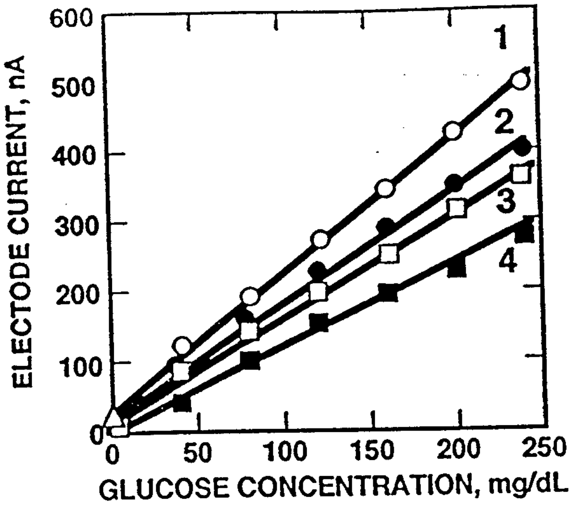

FIG. 4 Calibration curves for direct electrochemical (non-enzymatic oxidation) of glucose on electrodes coated with Stöber glass beads with different radius: 20 nm (1); 45 nm (2); 70 nm (3); 100 nm (4).

allows thin films to be fabricated using simple procedures. Although the deposition of particulate sols appears well-suited to the preparation of the inorganic membranes, problems associated with cracking, phase, and grain growth so far have thwarted attempts to prepare defect-free, supported membranes with the small pore sizes required to achieve molecular sieving. These techniques need further improvement.

Direct oxidation of glucose on the platinum surface even in the absence of glucose oxidase can be another reason for such behavior. The increase of the oxidation signal of the electrodes coated with 100 nm beads was higher than that for the electrodes coated with 20 nm beads. Because of the surface cracks (seen on Fig. 1), glucose will contact the electrode surface and more easily producing a direct oxidation signal.

Fig. 4 shows the calibration curves of a set of electrodes similar to those shown on Fig. 3.a but with no immobilized enzyme. The observed current signal in

TABLE 2. Comparison of the initial sensitivity of the electrodes with time

Day →	1	3	5	9	14	18	35	53
Diameter (nm) ↓	Initial Sensitivity (nA.dL/mg)							
20	14.8	9.75	8.75	4.00	3.75	2.50	3.00	
45	10.0	6.25	5.25	2.25	1.75	1.25	1.00	0.96
70	7.50	6.50	5.25	5.25	5.00	5.50	6.00	4.00
100	5.75	6.25	4.25	3.25	3.00	2.25	2.00	1.90

this case is due to direct electrochemical oxidation of glucose on the electrode surface. Comparison of Fig. 3.a and Fig . 4 illustrate the catalytic effect of the enzyme on glucose oxidation.

Life Time of the Sensors

Table 2 compares the sensitivity of the electrodes coated with Stöber glass layer with different bead radii as a function of the storage time. From the Table it can be seen that up to 5 days after immobilization, the value of the response did not change dramatically. The electrodes coated with 20 nm Stöber glass beads showed the highest response followed by that with 45 nm, 70 nm and 100 nm Stöber glass beads in the coating layer respectively. However, 2 weeks after immobilization, the response order changed: the electrode with 70 nm Stöber glass beads showed the highest response signal, followed by electrodes coated with 20 nm, 100 nm and 45 nm glass beads in that order.

Fig. 5 shows the life time curves of the sensors. From the Figure it can be seen that in the beginning of the testing period the response of the electrodes

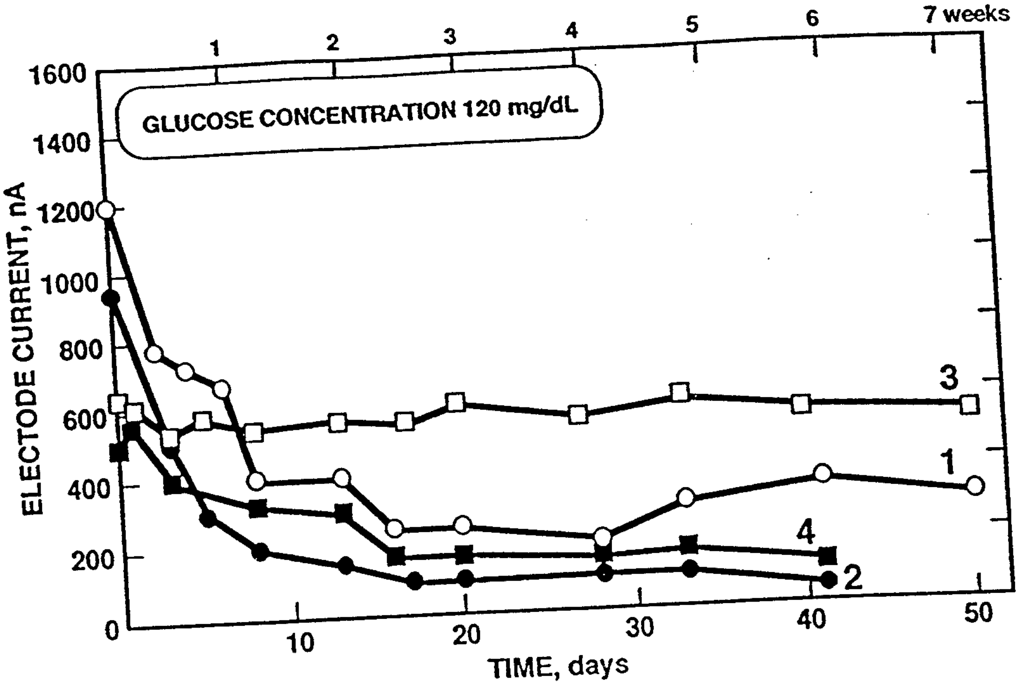

FIG. 5 Lifetime curves of the enzyme electrodes coated with Stöber glass beads with different radius: electrode signal at glucose concentration of 120 mg/dL as a function of electrode storage time.

decreased quickly, except for the electrode with 70 nm Stöber glass beads. A possible explanation of this could be some release during the first days of experiments of enzyme weakly bound to the glass beads. Deactivation (poisoning) of the platinum working electrode surface during the test by oxidation products could also contribute to the decrease in the overall electrode sensitivity.

After 10 days the responses tended to become stable. The electrode with 70 nm Stöber glass beads demonstrate the most stable long-term performance. It can be seen from the Fig. 5 that the signal of this electrode was stable over a 50 days period. The response sensitivity was within the acceptable limits during that period of testing. It is possible that the electrodes with 70 nm bead radius offer the optimum porosity and surface area for the enzyme immobilization. On the other hand, the electrode with 20 nm Stöber glass beads demonstrated highest initial signal values, but decreased to a lowest value of the response during the first week. This is probably because the cracks on this electrode surface were the largest. Hence, the barrier to hydrogen peroxide and glucose was smaller than that of other electrodes. Also, since the porosity was the smallest of the four electrodes, the enzyme penetration was the least. Therefore, the exposed enzyme on the surface of beads could have easily been washed off.

The electrode coated with Stöber glass with 45 nm bead radius showed the second greatest decrease. Electrodes coated with Stöber glass with beads of radius 100 nm were more stable than the electrodes coated with 20 nm and 45 nm beads. The possible reason may be the better uniformity of the layer and porosity. From the data we can see that the best porosity for enzyme immobilization was shown by the electrode with beads radius of 70 nm . The change in the order of response could have been due to the release of enzyme into the bulk solution either due to poor binding of enzyme onto the matrix, or due to the cracks in the glass coating layer.

Measurement of Glucose Concentration in Blood Plasma

The evaluation of the enzyme electrode performance in blood plasma can be used as an initial interference test. A calibration curve in bovine blood plasma is obtained by a procedure similar to that when phosphate buffer solution is used. Fig. 6 presents a comparison between the calibration curve in bovine blood plasma (curve 2) and two calibration curves in phosphate buffer solution, obtained before (curve 1) and after several hours of operation in undiluted blood plasma (curve 2) for an electrode with bea radius 70 nm . The calibration curve in bovine blood plasma starts from a concentration of glucose of 113 mg/dL (the actual concentration of the sample) to avoid dilution. An extrapolation of the calibration curve in blood plasma (the part of curve 2 shown by the dotted line) was used for

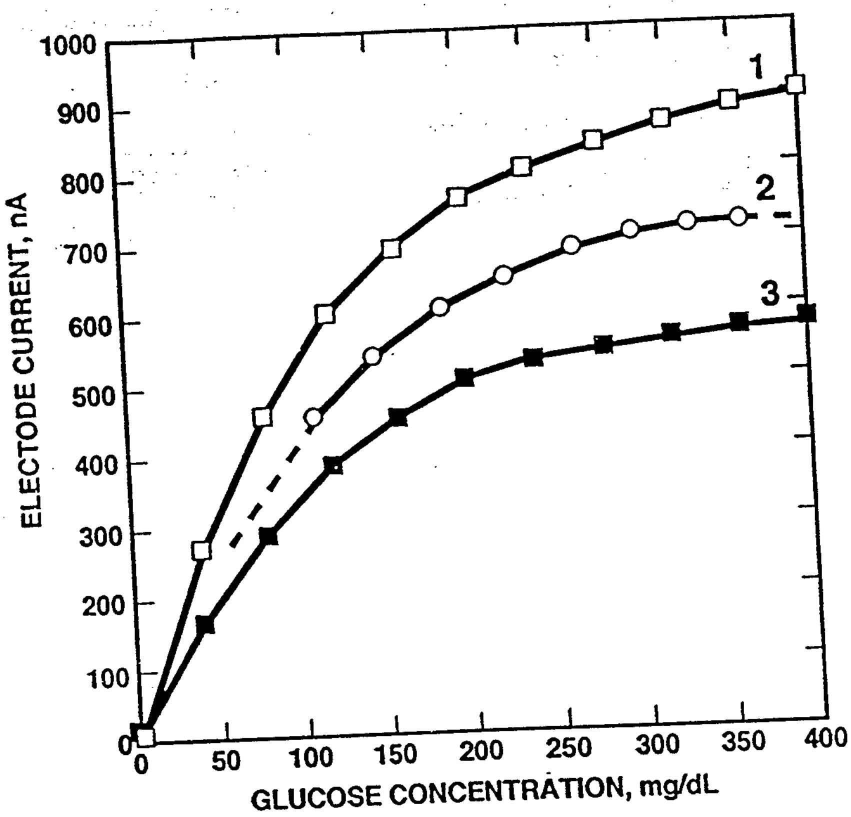

FIG. 6 Comparison of the calibration curve for the glucose electrode in bovine blood plasma (curve 2) with those in phosphate buffer (curve 1 before use and curve 2 after several hours of use in blood).

the estimation of glucose concentration lower than 113 mg/dL. Variation of the glucose concentration in the plasma is achieved by addition of small aliquot volumes of glucose stock solution to avoid dilution.

Glucose concentration in a serum sample (glucose level 113 mg/dL) has been monitored by the enzyme electrode for 6 hours (in vitro) with no noticeable change in the electrode response. During the following 12 hours of continuous glucose monitoring with the same electrode the signal decreased by 15 % of its initial value. After this test a calibration curve of this electrode in phosphate buffer glucose solution was obtained and the results are shown on Fig. 6 , curve 3.

The sensitivity of the electrode in blood plasma decreases by about 15 % with respect to the initial slope of the calibration curve obtained before the serum test (compare curves 1 and 2 in Fig. 6).

The decrease of glucose current response in undiluted serum and in phosphate buffer after the serum tests is probably caused by deposition of proteins from the plasma onto the glass bead coated electrode surface. This deposition can be consider irreversible because the electrode properties remain practically constant in tests (in phosphate buffer or in blood plasma) afterwards. It can be hypothesized that this deposition caused an increase of the diffusion limitation of dissolved oxygen and glucose leading to a decrease in the current signal.

Several whole bovine blood samples with known glucose concentrations (measured using routine clinical methods) were obtained. From these samples blood plasma was prepared by centrifugal separation. The amperometric response of the enzyme electrode with bead radius 70 nm, to the glucose levels in blood plasma was obtained and the concentration of glucose estimated using the electrode calibration curve in blood plasma (Fig. 6, curve 2). These glucose concentrations, measured by the electrode, are shown in Fig. 7 together with the data for the same samples measured by conventional method. The correlation between the two sets of measurements is characterized by the slope 0. 896 with a regression coefficient 0.983 (number of samples was 11).

CONCLUSION

The glass-coated needle-type electrode was made by using silanization followed by cross-linking the enzyme onto glass beads of four different sizes: 20 nm, 45 nm, 70 nm, 100 nm. Of the four electrodes, the electrode coated with Stöber glass with 70 nm bead radius showed the best characteristics in stability and

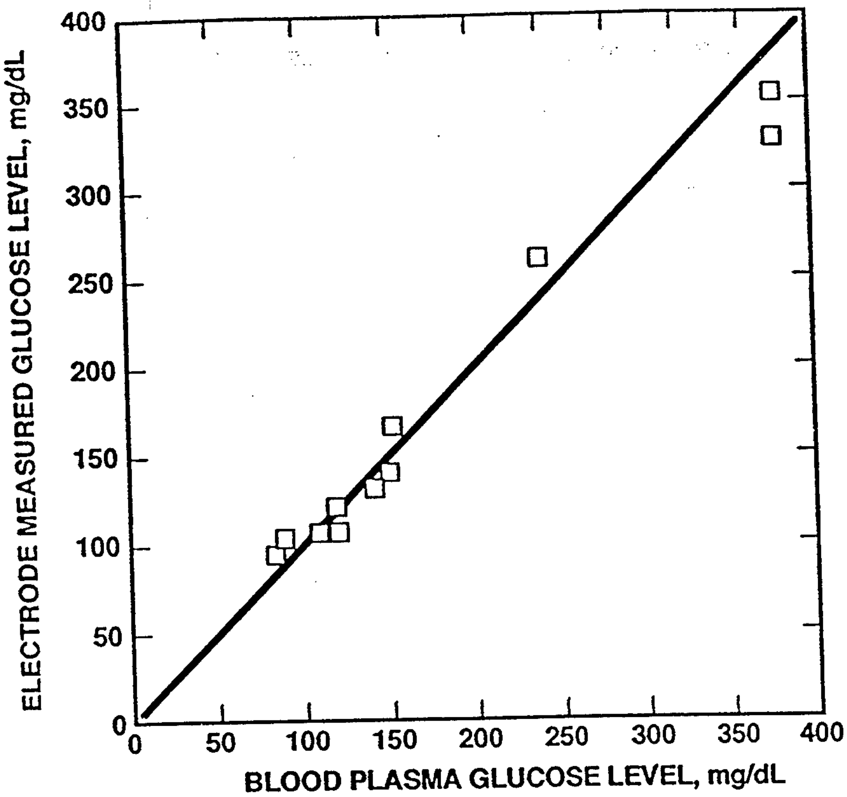

FIG. 7 Response of electrode to blood plasma glucose.

sensitivity. Preliminary tests of the enzyme electrode coated with 70 nm glass beads in undiluted blood plasma suggest that biosensors based on such electrodes can be used for glucose measurements in physiological fluids. Further experiments need to be performed to improve the performance of the biosensor. This includes improvement in the control of porosity, surface pretreatment and particularly avoidance of cracking of the Stöber glass layer.

ACKNOWLEDGEMENTS

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Our gratitude to Ms. Carol S. Ashley for the Stöber glass beads preparation.

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APPENDIX 7

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Mathematical Modeling of an Amperometric Enzyme Electrode Based on a Porous Matrix of Stöber Glass Beads

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Mathematical Modeling of an Amperometric Enzyme Electrode Based on a Porous Matrix of Stöber Glass Beads

Abstract: The mathematical model of a glucose sensor based on the amperometric detection of hydrogen peroxide using immobilized glucose oxidase (GOD) has been described. In this sensor GOD is immobilized on Stöber glass beads that are attached to a platinum electrode. The influence of the bead radius (r_b, ranging 20 nm, 45 nm, 70 nm, 100 nm and 200 nm) on the performance of the sensor has been analyzed. The total enzyme concentration defined per unit interfacial area increases directly with the bead radius and the effective diffusivity of the substrate in the enzyme layer decreases with increasing bead radius. The model describes approximate analytical solutions for the behaviour of the system, which is assumed to follow the Michaelis-Menten scheme of reaction. Two distributions of the enzyme in the bead layer have been taken into consideration in the discussion. Numerical solutions have also been presented to give a complete picture of the behaviour of the system. Comparison of numerical solutions and approximate analytical solutions suggests that the model is consistent in the regions of approximations. The model predicts different behaviour of the system on either side of the critical radius (approximately 26 nm). The process is essentially diffusion controlled for the sensors with beads of radius smaller than the critical radius and the current response of the sensors in this case increases with the increase in bead radius. The current response of the sensors with beads of radius greater than the critical radius, decreases with the increase in bead radius. The regime of operation (kinetic control or diffusion control) for this case depends on the value of the Thiele modulus.

Keywords: Amperometric Glucose sensor, Model, Stöber glass.

INTRODUCTION

The development and analysis of a mathematical model for an enzyme electrode is very crucial to the understanding of the behaviour of the electrode. Modeling can help improve the design of the electrode and recognize the role of various components involved in the response of the electrode and its behaviour. These components could be the enzyme loading, the enzyme activity, enzyme concentration (Sakamoto,1992; Yokoyama *et al.* 1991;), thickness of the membrane(Hameka & Rechnitz, 1983), method of immobilization(Lyons *et al.* 1992), diffusion characteristics of the membrane(Hameka & Rechnitz, 1983; Leypoldt & Gough, 1984;), substrate concentration(Iliev *et al.* 1992), influence of the mediator(Albery *et al.* 1992), kinetics of the enzyme reaction or the kinetics at the electrode surface and so forth.

Biosensors most frequently consist of enzyme electrodes where the enzyme is immobilized on the surface of the electrode or in a membrane surrounding the electrode. Glucose sensors, owing to their wide use in the field of medicine, are the most important class of biosensors. Amperometric techniques have been the most favoured method employed in the study of glucose sensors .

Amperometry involves measurement of the current when a constant potential is applied between the working electrode and the reference electrode. This current is related to the concentration of the substrate (analyte) in the bulk (sample) solution. Frequently, the enzyme glucose oxidase is immobilized on the surface of the electrode in a glucose biosensor. This enzyme catalyses the oxidation of glucose to gluconolactone and hydrogen peroxide. The increase in concentration of hydrogen peroxide or the decrease in concentration of oxygen during the progress of the enzymatic reaction can be monitored amperometrically. The dependence of the current on the analyte concentration is used for sensor calibration.

Development of a model for an enzyme electrode or a sensor involves deriving equations that can be used to simulate such calibration plots and make clear the role of various components involved in the working of the sensor. Modeling of a sensor involves describing the reaction scheme, analyzing the geometry of the system, describing the process by setting up equations, making model approximations and finally solving the equations to express the unknowns in terms of the knowns. The accuracy of the model will however depend on the degree of approximation and the validity of the approximations.

Typically, a model would involve setting up of differential equations or difference equations or other forms of representation depending on the type of the model employed. The model can contain explicit solutions (Hameka & Rechnitz, 1981; Iliev et al. 1992; Leypoldt & Gough, 1984;) or numerical methods(Hameka & Rechnitz, 1983; Tetsu *et al.* 1992; Yokoyama *et al.* 1992; Yokoyama *et al.* 1991). Some work (Bartlett & Pratt, 1993; Schulmester, 1990;) on modeling throw light on the techniques involved.

This paper describes the development of a model to analyze the performance of a glucose sensor with immobilized GOD enzyme. The enzyme is immobilized on sol-gel derived Stöber glass beads that are attached to a platinum electrode. Five different sizes of the beads (20 nm, 45 nm, 70 nm, 100 nm, 200 nm) have been considered in the study. The model seeks to evaluate the performance of the sensor as a function of the bead radius.

COMPUTATIONAL METHODS

The numerical solutions for the differential equations were obtained using a finite difference method. In this finite-difference method, the derivatives were replaced by the central difference approximations obtained from the difference between Taylor's forward and backward difference approximations. A series of equations involving the independent and dependent variables were later set up. The range of the independent variable was divided into four equal intervals. These equations were solved by running a program on the software package 'Mathematica' - [Version 2.2 of the Wolfram Research Inc., U.S.A.] on a Macintosh.

THE MODEL

Reactions

The enzyme glucose oxidase catalyses the reaction of glucose and oxygen giving hydrogen peroxide and gluconolactone as given below. In the system described, there are no artificial mediators.

The reaction scheme is as follows:

$$\beta\text{-D-Glucose} + \text{GOD}_{(\text{oxidised state})} \text{ -------> Gluconolactone} + \text{GOD}_{(\text{reduced state})} \quad (1)$$

$$\text{GOD}_{(\text{reduced state})} + \text{Oxygen} \text{ -----> } H_2O_2 + \text{GOD}_{(\text{oxidised state})} \quad (2)$$

The overall reaction is thus,

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{Gluconolactone} + H_2O_2 \quad (3)$$

The reaction mechanism for the above reacting system (Reactions (1), (2) and (3)) is given by the ping-pong bi-bi mechanism for two substrates (Schulmester, 1990) and the rate of the reaction (rate of disappearance of the substrate (glucose)) is given by:

$$\text{Rate} = -k\,[E]/(\,(K_m/[S]) + (K_{O2}/[O_2]) + 1) \qquad (4)$$

where

k - is the rate constant of the enzymatic reaction,

$[E]$ - is the total enzyme concentration in the bead layer,

$[S]$ - is the substrate concentration in the layer,

K_m - is the Micahelis-Menten constant for the substrate(glucose),

$[O_2]$ - is the concentration of oxygen and

K_{O2} - is the Michaelis-Menten constant of the other substrate, Oxygen.

The rate of appearance of hydrogen peroxide will be the same as above, but with a positive sign. The hydrogen peroxide produced is oxidized on the surface of the electrode (Pt) as follows:

$$H_2O_2 \xrightarrow{\text{Platinum}} 2H^+ + O_2 + 2e^- \qquad (5)$$

The model describes a glucose sensor that is based on the amperometric detection of hydrogen peroxide produced in the enzymatic reaction. The flux of hydrogen peroxide at the surface of the electrode is used to link the current measured and the substrate concentration in the bulk.

Fig.1 is a schematic of the platinum electrode and the arrangement of glass beads. The enlargement illustrates the Stöber glass beads attached to the electrode and the assumed close packed hexagonal structure. Glucose (substrate) diffuses from the bulk (at R=1) and reacts with the enzyme (GOD) in the enzyme layer of thickness L.

This is a typical reaction-diffusion phenomenon. Hydrogen peroxide produced in the reaction reacts at the electrode surface (R=0) according to reaction (5), giving off electrons. The flux of hydrogen peroxide at the surface of the electrode (R=0) is related to the current measured.

The electrode system described here has a cylindrical geometry (Fig. 1). It is assumed that there is axial symmetry and that there is no concentration variation along the z-direction. The development of transport equations for the substrate and H_2O_2 in the enzyme layer leads to the following equations in cylindrical coordinates under steady state conditions.

$$D_S [\ d^2[S]/dr^2 + (1/r)(d[S]/dr)\ ] = k[E]/[\ (K_m/[S] + (K_{O2}/[O_2]) + 1] \qquad (6)$$

$$D_H [\ d^2[H]/dr^2 + (1/r)(d[H]/dr)\ ] = -k[E]/[\ (K_m/[S] + (K_{O2}/[O_2]) + 1] \qquad (7)$$

D_S and D_H are the effective diffusivities of the substrate and hydrogen peroxide respectively and **[H]** is the concentration of hydrogen peroxide. The underlying assumptions are :

1. The concentration of oxygen $[O_2]$ in the system is high enough so that the change in its concentration due to the consumption in the reaction can be neglected.
2. The rate equations in equation (4) is approximated to the Michaelis-Menten equation

$$\text{Rate} = k[E][S]/(K_m + [S]) \qquad (8)$$

This happens when $(K_m/[S] + 1) >> K_{O2}/[O_2]$.

3. The Stöber glass beads are arranged in a regular hexagonal closed-packed

The Enzyme Layer

This is the layer of thickness L surrounding the electrode and containing glucose oxidase immobilized on Stöber glass beads (Fig. 1). This layer of glass beads is treated as a medium (porous matrix) by itself and the following analysis used.

As could be seen from equation (6), D_S, the effective diffusivity of substrate in this medium of beads and [E], the total enzyme concentration need proper interpretation and evaluation as they are dependent on the parameters of the bead layer, especially the bead radius.

Enzyme Concentration

The term [E] in equation (6) is in fact the enzyme concentration per unit volume of the reacting system and in this model, it has been adapted to suit the nature of the electrode system. Essentially, the enzyme concentration that will be used in this model is the concentration per unit interfacial area of the packing, since the latter is an intrinsic property depending on the particular type of packing. Hence, we introduce another term a, the interfacial area per unit volume of the packing. The enzyme concentration varies linearly with the bead radius.

Effective Diffusivity

The presence of beads in the layer offers resistance to diffusion of substrate and this decreases the diffusivity of the substrate in the layer (D_S) compared to the diffusivity in the bulk. D_S is a function of bead radius and packing. A general equation describing this given by (Satterfield, 1970)

$$D_{eff} = D_{media} (\varepsilon/\tau) \tag{9}$$

where ε is the volume porosity and τ is the tortuosity of the packing. Of these, the volume porosity is a function of the packing only and the tortuosity is also a function of bead radius. Based on the geometrical considerations of the hexagonal packing and using the fact that the thickness of the bead layer is much less than the diameter of the electrode, a simple relation has been derived.

$$D_{eff} = D_{media} (2 L) (6/r_b)(\varepsilon) [(3)^{1/2} - (\pi/2)]/[24 (2)^{1/2} \pi] \tag{10}$$

where, L is the thickness of the enzyme layer and r_b is the radius of the bead and D_{eff} is the diffusivity of the substrate in the medium of glass beads and D_{media} is the diffusivity of the substrate in the bulk. Fig. 2 illustrates this relation. This figure is a graph of equation (10) showing the variation of the effective diffusivity with the bead radius. Based on the discussion so far, it is clear that the modified form of equation (6) which is relevant is

$$D_S [d^2[S]/dr^2 + (1/r) (d[S]/dr)] = k a [E] [S]/(K_m + [S]) \tag{11}$$

a being the interfacial area per unit volume of the packing. The following paragraphs present a simple analysis of equation (11). It should be noted that equation (7) can be modified to yield

$$D_H [d^2[H]/dr^2 + (1/r) d[H]/dr] = -k a [E] [S]/(K_m + [S]) \tag{12}$$

On solving these two equations simultaneously, the current measured is related to the flux of H_2O_2 at the surface of the electrode and hence to the concentration of the analyte in the bulk. This involves application of certain boundary conditions as in Appendix A.

Approximate Model

An explicit analytical solution to equation (11), though it exists (Kernevez, 1980), is difficult to find and most treatments of such a nonlinear equation involve numerical solutions. Solution to equation (11) in rectangular coordinates and one dimensional approximation (Hameka & Rechnitz, 1981) also has been derived only for certain approximate conditions. A satisfactory treatment of such reaction-diffusion systems can be found elsewhere (Ovchinnikov *et al.* 1989). The basic assumptions for the treatment in this section are:

1 Enzyme is distributed homogeneously in the enzyme layer i.e. enzyme concentration is not a function of the position in the layer.

2 Since the thickness of the enzyme layer is much less than the diameter of the electrode, the system is approximated to rectangular coordinates, i.e., to one dimensional variation of the concentration. Equations (11) and (12) become

$$D_S\,(d^2[S]/dr^2) = k\,a\,[E]\,[S]\,/\,(K_m + [S]) \qquad (13)$$

$$D_H\,(d^2[H]/dr^2) = -k\,a\,[E]\,[S]\,/\,(K_m + [S]) \qquad (14)$$

These equations, when non-dimensionalized, using dimension-less variables,

$C_S = [S] \;/\; (K_m)$

$C_H = [H] /(K_m)$

and $R = (r - d)/\,(L)$, yield

$$D_S\ (d^2C_S/dR^2) = (L^2\,k\,a\,[E]/K_m)\ [C_S/(C_S + 1)] \qquad (15)$$

$$D_H\ (d^2C_H/dR^2) = -(L^2\,k\,a\,[E]/K_m)\ [C_S/(C_S + 1)]. \qquad (16)$$

We consider equation (15) for the development of the approximate model. Rearranging terms in equation (15) we get

$$(d^2C_S/dR^2) = \beta\ [C_S/(C_S + 1)] \qquad (17)$$

where β is a dimension-less group - the Thiele modulus. It gives the ratio of the influence of kinetics of the reaction to the influence of diffusion of the reactants and products on the system.

Large values of this group, β, will indicate diffusion limitations and small values will indicate kinetic controlled conditions of the system. The value of β is directly dependent on the enzyme loading and clearly depicts that high loading favours diffusion controlled regime.

The above equations have been analyzed and solutions presented for two different limiting cases (Bartlett and Whitaker, 1987), namely

Case 1. $C_S \gg 1$ and

Case 2. $C_S \ll 1$.

However, the boundary conditions and the final solutions are given here for sake of completeness. The boundary conditions are :

1. At $R = 0$; $dC_S/dR = 0$ and $C_H = 0$ (18) and
2. At $R = 1$; $C_S = C_S^b$ and $C_H = 0$ (19)

C_S and C_H are the dimensionless concentrations of the substrate and H_2O_2.

In amperometric detection, the equation describing the current measured is given by

$$I = n\ F\ A\ D_H\ (d[H]/dr)|_{r=d} \qquad (20)$$

where, I is the current

n is the number of electrons transferred

F is the Faraday's constant

A is the surface area of the electrode and

$D_H(d[H]/dr)|_{r=d}$ is the flux of H_2O_2 at the electrode surface per unit time.

The expression for the current for the case 1, becomes

$$I = (n F A K_m D_S \beta)/(2 L) \qquad (21)$$

and for case 2, it becomes

$$I = n\ F\ A\ (1 - \mathrm{sech}[(\beta)^{1/2}])\ (D_S / L)\ (S_b) \qquad (22)$$

It should be noted that equation (22) is linear in bulk substrate concentration. Same methods of analysis described above can be extended to other distributions of enzyme in the layer. The analysis for a distribution of enzyme varying exponentially with the position in the layer is given below.

The enzyme concentration [E] in equation (15) could be considered to be

$$[E] = [E_0]\ e^{R\alpha} \qquad (23)$$

Then for Case 1, under conditions of $\alpha = 1$

$$(d^2C_S/dR^2) = \gamma\ e^R \qquad (24)$$

where, $\gamma = (k \lambda L) / (2 \pi h K_m D_S (L + d(e - 1)))$ (25)

$[E_0]$ is found by integrating expression (23) over the whole volume of the enzyme layer and equating the total amount of enzyme in the layer to the total amount of enzyme loaded.

λ	being the total enzyme loading
h	being the height of the electrode on which enzyme has been immobilized and
e	being the natural logarithm base
α	being an index

Equation (24) leads to the following equations when solved using the boundary conditions (18) and (19)

$$C_S = \gamma e^R - \gamma R + (C_S^b - \gamma(e - 1)) \tag{26}$$

$$C_H = (D_S \gamma / D_H)(R(e - 1) - e^R + 1) \tag{27}$$

$$I = (n F A K_m D_S \gamma (e - 2)) / (L) \tag{28}$$

Using equation (25) and (28), we can see the dependence of the current on the thickness and the diameter of the electrode as well. It is clear that with increasing values of both the thickness and the diameter of the electrode, the flux of hydrogen peroxide at the electrode, and hence the current, decreases.

Apparently, as the value of α in equation (23) increases, the profile of enzyme concentration in the layer becomes steeper. So, for large values of this parameter, more of the enzyme tends to get concentrated at the interface of the bead matrix with the bulk, i.e., R = 1 in Fig. 1. Also, α will be a function of the bead size, because, the penetration of enzyme molecules into the matrix will be greatly influenced by the bead radius. For larger sizes, there will more penetration and α will be small and it will be large for smaller bead sizes. Also, the dependence of α with bead size will reduce with increase of bead radius.

The approximate model should consider the case when the reaction takes place on the surface of the enzyme layer, i.e., at the interface with the bulk (R = 1 in Fig.1). This represents the case when the rate of the reaction is very fast compared to the diffusional transport of the substrate in the enzyme layer. This happens for electrodes with beads of radius lesser than the critical radius and also when the value of β is very large, i.e., when diffusion limitations are dominant. The critical radius can be defined as that value of the bead radius below which the packing does not allow the enzyme molecules to penetrate into the packing. This happens for large values of α.

For such a treatment, no transport equations are set up, rather the rate of the reaction is used to derive the expression for the current. The rate of the enzymatic reaction is given by the Michaelis-Menten equation [equation (8)] and the flux is determined as the gradient of the hydrogen peroxide concentration profile at the surface of the electrode.

The current expressions are:

$$I = n F \lambda k ([S] / ([S] + Km)) \qquad (29)$$

for electrodes with beads of radius 20 nm, and

$$I = (470 / a) [S] / ([S] + Km) \qquad (30)$$

for the other electrodes (bead radius = 45 nm, 70 nm, 100 nm and 200nm).

The derivations are given in Appendix A. In the range of bead radius investigated, there is a 'critical radius' of the bead on either side of which the model behaves differently. The above analysis of the diffusion-controlled regime show by geometrical considerations that this value lies around 26 nm.

The K_m value assumed is closer to the true K_m value (i.e. the K_m for the enzymatic reaction in solution). Therefore, when treating the system on either side of the critical radius, different values of K_m are used.

Numerical Solutions

Assuming an exponential distribution ($\alpha = 1$) of the enzyme in the bead layer, we get the following equation in cylindrical coordinates under steady-state conditions.

$$d^2C_S / dR^2 + \{1/[R + (d/L)]\}dC_S/dR = (L^2 k a [E_0] / K_m D_S)[e^R C_S/(C_S + 1)] \quad (31)$$

This equation was solved numerically using the finite-difference approximation after obtaining $C_S|_{R=0}$ using methods described elsewhere (Yokoyama *et al.*, 1992). The current values for different bulk concentrations of the substrate were determined. For a homogeneous distribution of enzyme, equation (31) takes the form

$$d^2C_S / dR^2 + \{1/[R + (d/L)]\} dC_S/dR = (L^2 k a [E] / K_m D_S)[C_S/(C_S + 1)] \quad (32)$$

This has to be solved numerically in a similar fashion.

RESULTS AND DISCUSSION

The calibration plots obtained from numerical solutions (Fig. 3 and Fig. 4) show the behaviour of the system in accordance with Michaelis- Menten scheme of reaction. The plot of equations (29) and (30) (Fig 5) shows a reverse behaviour of the system compared to the other calibration plots, as the model suggests, except for the anomaly of the behaviour of the electrode with 20 nm bead radius. The method of derivation of equation (29) and the definition of enzyme concentration therein throw light on this. In describing the rate of the enzymatic reaction, the enzyme is assumed to exist in a monolayer, but because of the size of the beads (20 nm- lesser than the critical radius) and loading of enzyme, there might be more than one layer present. Also, only the outer most layer might be driving the reaction to completion rather than the participation of all the layers. The flux of hydrogen peroxide into the bulk has been neglected. This flux could be more than half of the values plotted because the diffusivity of hydrogen peroxide in the bulk is greater (equation (9)) than that in the enzyme layer (porous glass matrix).

Therefore, the enzyme concentration will approximately be reduced by a factor inversely proportional to the number of layers. The effect of this has been demonstrated by plotting such a variation, as in Fig. 6. This figure shows the variation of current with the number of layers for different substrate concentrations in the bulk.

There are certain distinct characteristics of the system and the model which need special attention. It is quite an obvious choice to consider a hexagonal packing for the beads, though a factor proportional to the probability of such a packing has to considered. As the size of the beads reduces, the degree of control on the properties of the glass bead layer (especially the arrangement of glass beads) also reduces. Therefore the validity of neglecting such a factor depends only to a very less extent on the procedure of coating adopted for the glass beads. Also, such a factor should accommodate the abnormalities of the coating as well.

The determination of effective diffusivity for the substrate in the packing again should take into account the probability of movement of the molecules in different tortuous channels created by the packing. Nevertheless, the flux (net) considered is in one direction (towards the center of the electrode). The model assumes a uniform flow area through the channels though in reality, the flow area between two successive layers might pass through a maximum in between. This might necessitate an averaging procedure of the flow area. But, the dimensions of the bead radius let us neglect the difference between such an average flow area and the uniform flow area. Again the packing is considered to be isotropic. The degree of anisotropicity will result in the probability factor for the packing discussed earlier.

As had been shown, there is different behaviour of the system on either side of the critical radius. The K_m values assumed are different on these regions. When the system is under diffusion-control (as in 20 nm case), the reaction is identical to that taking place in a homogeneous solution and the true value of K_m applies and when there is diffusion and reaction occurring simultaneously (as for the other electrodes), the apparent value has to be assumed. Thus, a model assuming $K_m = 15$ mM for the electrodes with 20 nm bead radius and $K_m = 6.5$ mM for the others, namely, 45nm, 70nm, 100nm and 200nm bead radius electrodes, should ideally represent the response and behaviour of the overall performance of the sensor.

CONCLUSION

A mathematical model describing the performance of an amperometric glucose sensor has been presented. The sensor uses glucose oxidase (GOD) immobilized on Stöber glass beads attached to a platinum electrode. The model predicts the existence of a 'critical bead radius' on either side of which, the behaviour of the sensor is different, suggesting different K_m values for different bead radii. Comparison of the numerical solutions and the solutions obtained from approximate model show consistent behaviour of the model in the regions of approximation.

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APPENDIX

Appendix A

Diffusion controlled reaction system

In this case, the reaction is assumed to occur completely on the surface of the enzyme layer at the interface with the bulk and the flux of H_2O_2 into the bulk (i.e., region where R>1 in Fig 1) is neglected.

For the 20 nm case, the enzyme concentration is simply expressed per unit outer surface area of the layer.

$$[E] = (\lambda / 2\pi (d+L) h) \quad \text{(A1)}$$

and the rate of the reaction is given by equation (8).

Since the rate at which the reaction proceeds is the rate at which hydrogen peroxide arrives at the electrode ,

$$A D_H \, d[H]/dr = 2\pi (d+L) h [E] \; k \; [S] \; /([S] + K_m) \quad \text{(A2)}$$

Therefore the current $I = n F A \; D_H \, d[H] / dr$ (A3)

is $n F \lambda k [S] / ([S] + K_m)$ (A4)

For the other electrodes, since there will be some penetration of the enzyme into the enzyme layer, and since an exponential profile is most likely, the concentration of the enzyme at the outer surface is found from the function

$$[E] = [E_0]\ e^R \qquad \text{(A5)}$$

Since the concentration expressed above is per unit interfacial area of the packing, it is adapted to the form of concentration per unit outer surface area. The conversion factor for hexagonal packing is **1.813** i.e. $\pi/(3)^{1/2}$ and hence the current equation using (A3), (C5) and (C2) becomes

$$\mathbf{I = n\,F\,A\ 2\pi\,(d+L)\,[\pi/(3)^{1/2}]\ k\ e\,[E_0]\,[S]\,/\,(\,[S]+K_m)} \qquad \text{(A6)}$$

$$\text{where } [E_0] = \lambda\,/\,2\,\pi\,h\,L\,a\,(\,1+d\,(\,e-1)\,) \qquad \text{(A7)}$$

$$\text{and hence } \mathbf{I} = (\,\mathbf{470\,/\,a}\,)\ [S]\,/\,(\,[S]\ +K_m\,) \qquad \text{(A8)}$$

Appendix B

Derivation of the expression for the effective diffusivity in the enzyme layer.

The whole layer of glass beads is considered to be a medium and a change in diffusivity occurs primarily because of the change in flow area when moving from the bulk into the layer. Other factors that affect, are the nature of the packing , porosity and the size of the beads.

Thus, if the bulk is considered to be medium **I** and the enzyme layer to be medium **II**, then, a concentration change $\delta[S]$ in medium **II** is equivalent to change $\varepsilon\delta[S]$ in medium **I** and similarly a distance of $2r_b$ in **II** is equivalent to πr_b in **I**. Here, [S] is the concentration of the substance (substrate) diffusing, r_b is the radius of the glass bead and ε is the volume porosity of the enzyme layer.

Thus, equating a particular mass flux through these two media with no accumulation at the interface, we get

$$D_{eff} = D_{media}\,\text{(flow area per unit volume of the packing) x (Volume of the packing) x } \{(2\varepsilon)/[(\pi) \text{ x (flow area in the bulk)}]\} \qquad \text{(B1)}$$

D_{eff} is the diffusivity in the enzyme layer and

D_{media} is the diffusivity in the bulk.

Therefore, for a hexagonal close-packed structure, the above formula reduces to

$$D_{eff} = D_{media}\,(2\,L)\,(6/r_b)(\varepsilon)\,[(3)^{1/2} - (\pi/2)]/[24\,(2)^{1/2}\,\pi] \qquad \text{(B2)}$$

NOMENCLATURE

$\mathbf{a}$ - Interfacial area per unit volume of the packing (nm^{-1})

$\mathbf{A}$ - Surface area at the electrode surface (nm^{2})

$\mathbf{C_H}$ - Dimension-less concentration of H_2O_2

$\mathbf{C_S}$ - Dimension-less concentration of the substrate

$\mathbf{C_S^b}$ - Dimension-less concentration of the substrate in the bulk

$\mathbf{d}$ - The radius of the electrode (nm)

$\mathbf{D_H}$ - Effective diffusivity of H_2O_2 in the enzyme layer ($nm^2\ s^{-1}$)

$\mathbf{D_{media}}$ - Diffusivity of the substrate in the bulk of the analyte ($nm^2\ s^{-1}$)

$\mathbf{D_S}$ - Effective diffusivity of the substrate in the enzyme layer ($nm^2\ s^{-1}$)

$\mathbf{[E]}$ - Enzyme concentration in the enzyme layer (moles nm^{-3})

$\mathbf{F}$ - Faraday's constant (coloumbs/mole)

$\mathbf{[H]}$ - Hydrogen peroxide concentration (moles nm^{-3})

$\mathbf{I}$ - Current measured (micro amperes)

$\mathbf{k}$ - Enzyme reaction rate constant (s^{-1})

$\mathbf{K_m}$ - Michaelis-Menten constant for the substrate (moles nm^{-3})

$\mathbf{K_{O2}}$ - Michaelis-Menten constant for the other substrate oxygen (moles nm^{-3})

$\mathbf{L}$ - The thickness of the bead layer (nm)

$\mathbf{n}$ - Number of electron transfer at the surface of the electrode

$\mathbf{[O_2]}$ - Oxygen concentration (moles nm^{-3})

$\mathbf{R}$ - Dimension-less distance in the cylindrical coordinates

$\mathbf{r}$ - Distance along the radial direction in the cylindrical coordinates (nm)

$\mathbf{r_b}$ - Radius of the bead (nm)

$[S]$ - Substrate concentration (moles nm^{-3})

$[S_b]$ - The substrate concentration in the bulk (moles nm^{-3})

α - An arbitrary index used in the enzyme distribution function

β - **Dimension-less group ($L^2 k a [E] / K_m D_S$)**

γ - Dimension-less constant used to describe the zero order reaction for an exponential distribution of the enzyme

ε - The volume porosity of the packing of the beads

τ - The tortuosity of the packing of beads

λ - The total loading of enzyme in the layer (moles)

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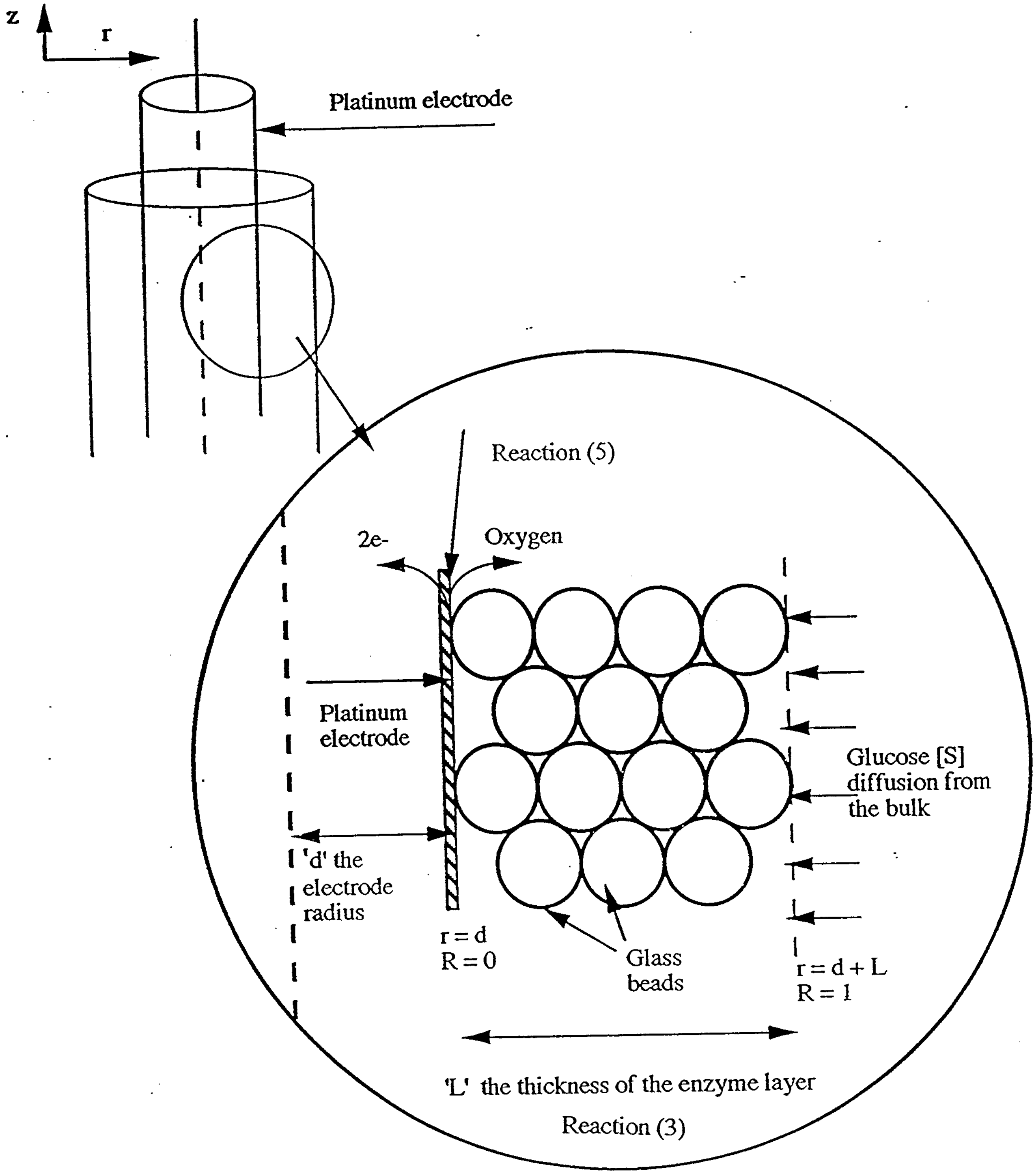
Figure. 5 Calibration plot obtained assuming the reaction to occur on the surface (diffusion control) of the enzyme layer

Figure. 6 Variation of current with the number of layers of enzyme on the outer surface, for the electrode with beads of radius 20nm



z
r
Platinum electrode
Reaction (5)
2e-
Oxygen
Platinum electrode
Glucose [S] diffusion from the bulk
'd' the electrode radius
r = d
R = 0
Glass beads
r = d + L
R = 1
'L' the thickness of the enzyme layer
Reaction (3)

Figure 1. Schematic of the electrode and the arrangement of the glass beads

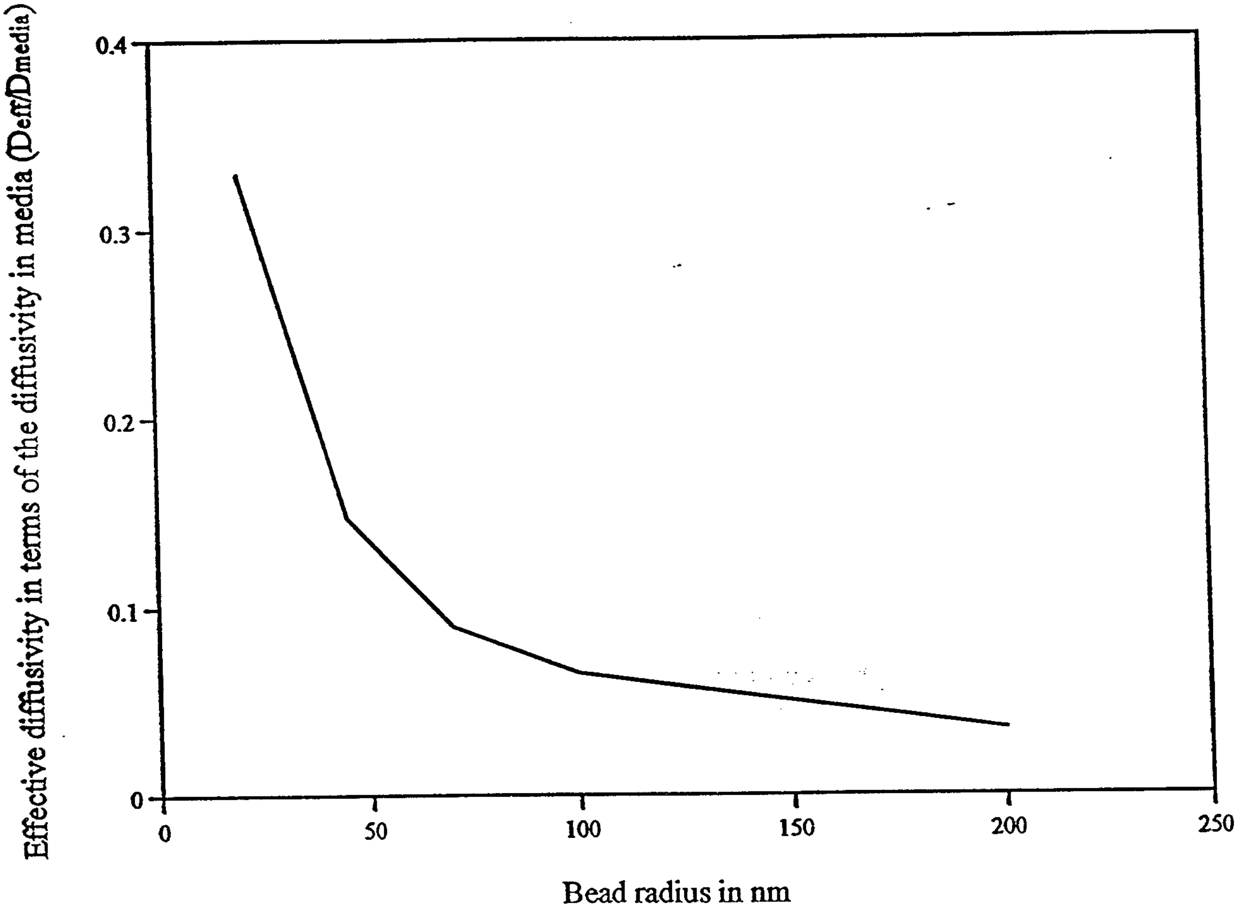


Figure. 2 Variation of the effective diffusivity with bead radius [Equation 10].

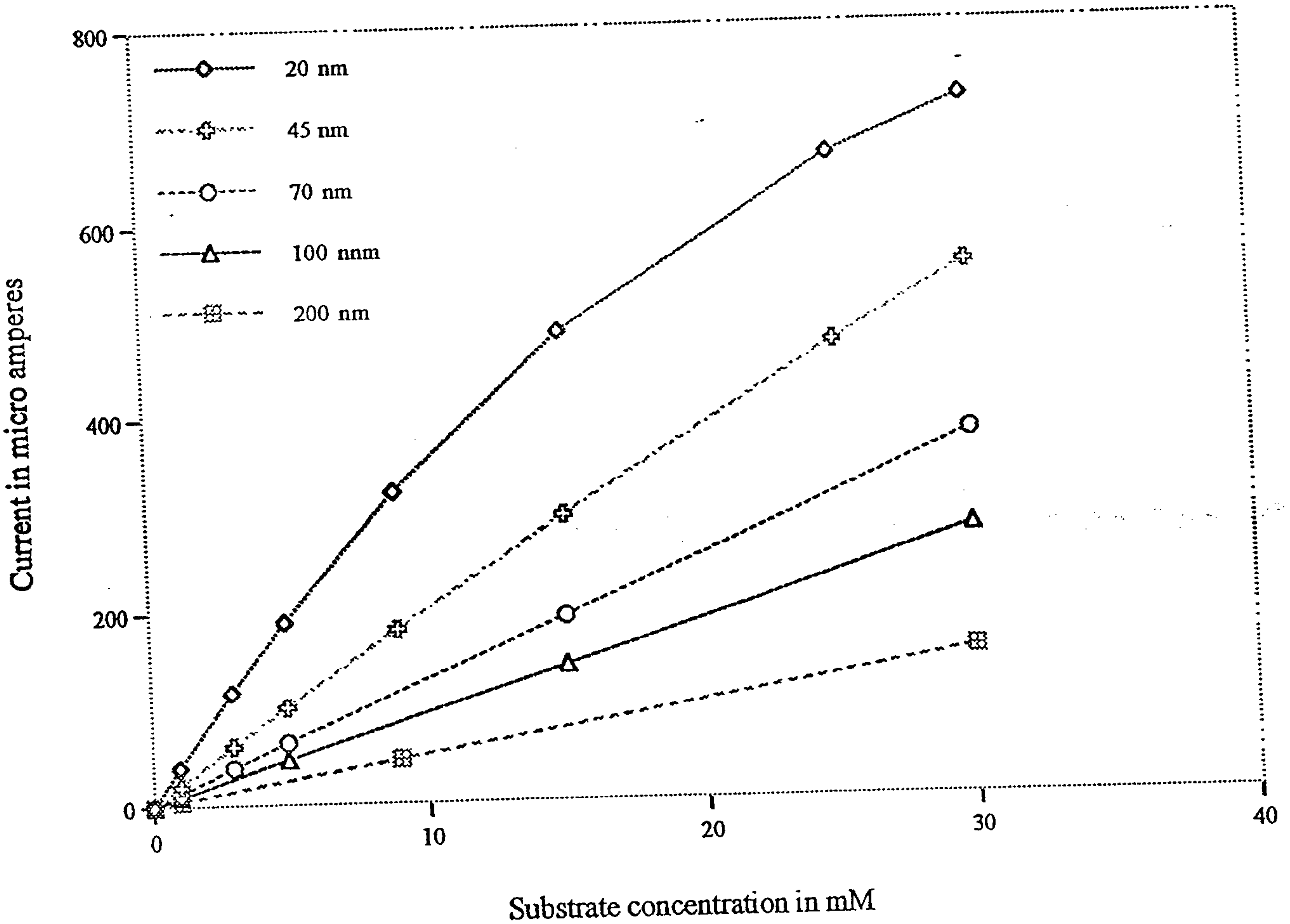


Figure. 3 Calibration plot obtained from numerical solutions for a homogeneous distribution of enzyme [Equation 32].

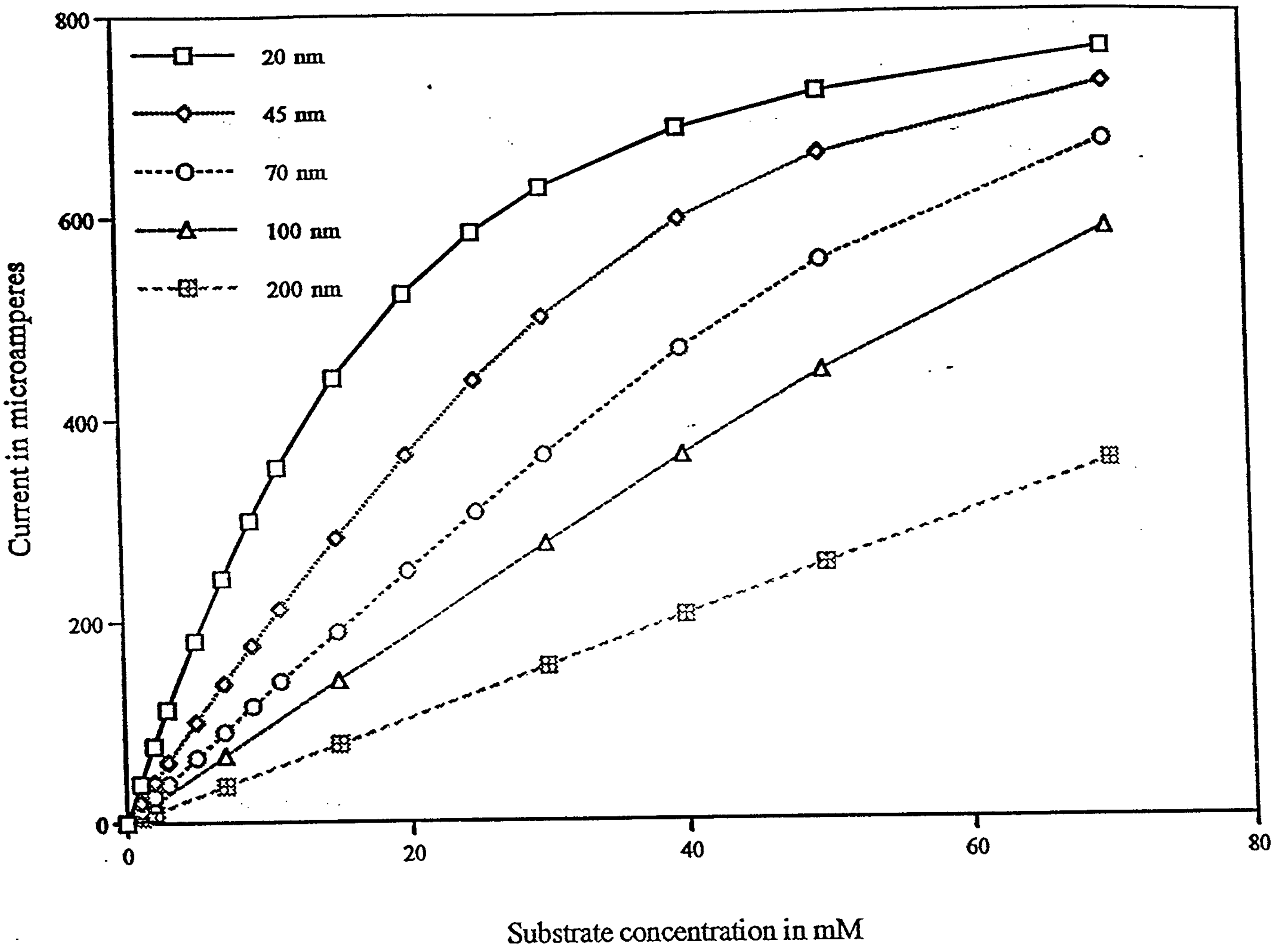


Figure. 4 Calibration plot obtained from numerical solutions for an exponential distribution of enzyme [Equation 31].

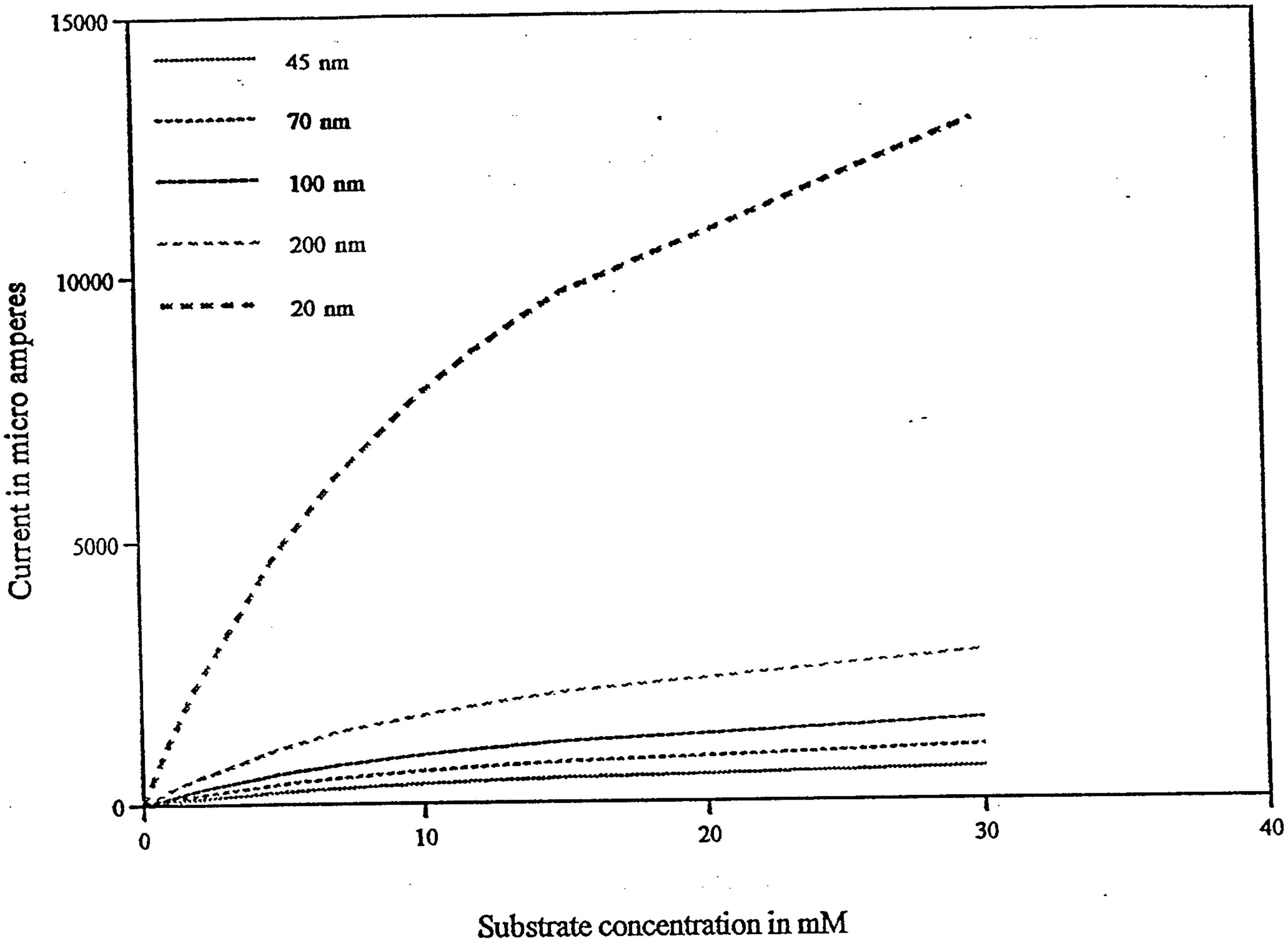


Figure. 5 Calibration plot obtained assuming the reaction to occur on the surface (diffusion control) of the enzyme layer [Equation 29].

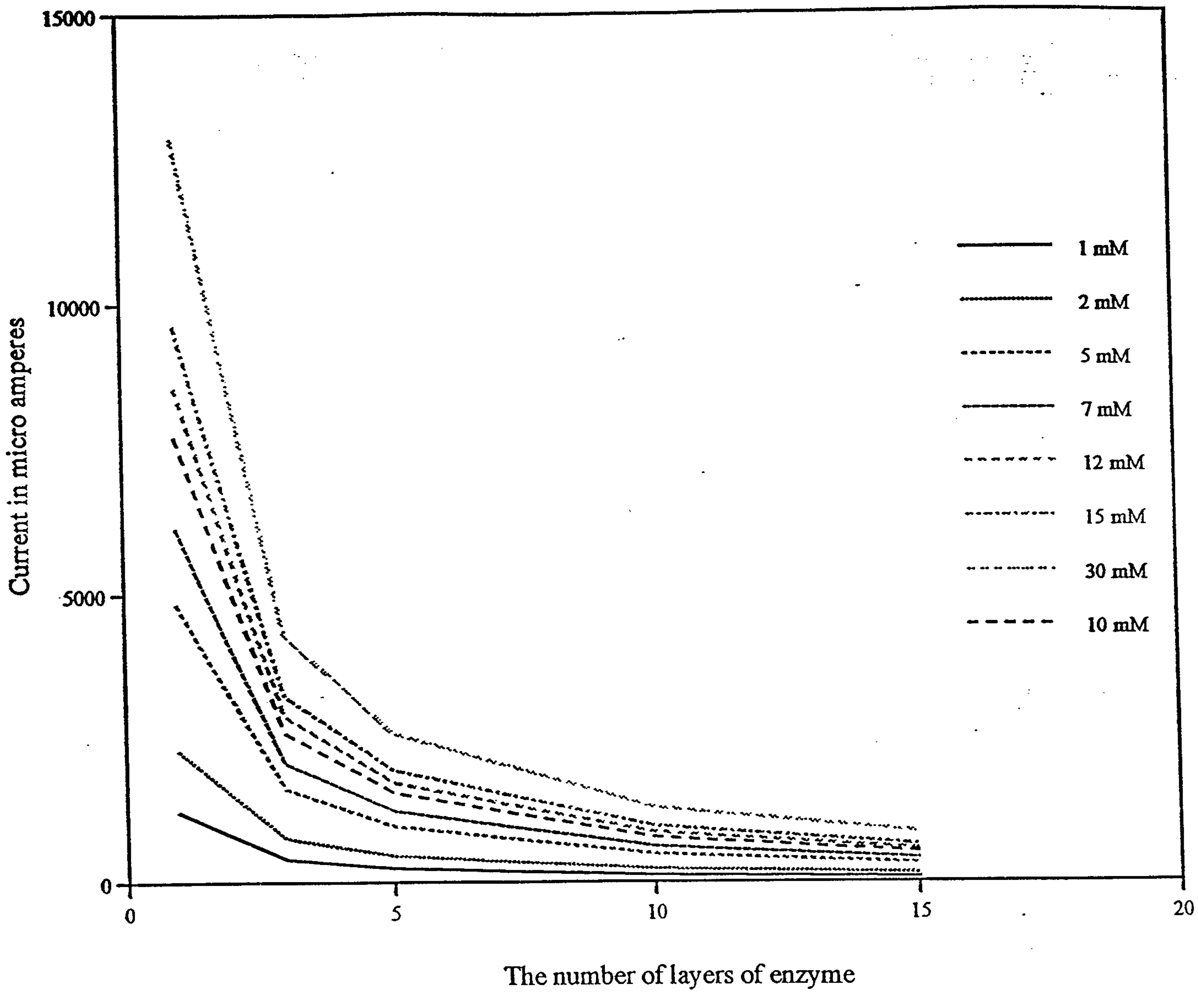


Figure. 6 Variation of current with the number of layers of enzyme on the outer surface, for the electrode with beads of radius 20 nm.

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17 October 1995

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Dear Dr Wilkins

Re: Biosensors & Bioelectronics

I am writing to confirm that your paper 'Mathematical Modeling of an Amperometric Enzyme Electrode Based on a Porous Matrix of Stöber Glass Beads' has been accepted for publication in the above mentioned journal.

You will receive proofs of your article along with copyright and reprint order forms in due course.

Yours sincerely

Matt Howells

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Team Leader
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